Synthesis and Function of Germination Stimulants for Seeds of the Parasitic Weeds *Striga* and *Orobanche* spp

Een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen, Wiskunde en Informatica

PROEFSCHRIFT

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Omslag: Electron Micrograph of fractioned *Striga hermonthica* seed showing the embryo and part of the seed coat.

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CHAPTER 1

Introduction

1.1 Background

The angiosperms *Striga* and *Alectra* (scorphulariaceae), and *Orobanche* (Orobanchaceae) are root parasitic plants which have a devastating impact on their host. Unlike almost all higher plants, the germination of seeds of root parasites depends on the receipt of a chemical signal (germination stimulant). These seeds germinate only in response to specific chemicals present in the rhizosphere of host and some non-hosts plants.[1,2] These parasitic flowering plants are entirely dependent on a specific association with the host that provides them with nutrients and water. The recognition system ensures that germination starts only when suitable host roots are available in the immediate vicinity.[3,4] *Striga* and *Orobanche* parasitize important food crops. For example, *Striga hermonthica* (Del.) Benth. and *Striga asiatica* (L.) Kuntze attack preferentially monocotyledonous crops such as maize, sorghum, millet and rice, in tropical and sub-tropical areas in Africa and Asia (especially India). Another important species, *viz*. *Striga gesnerioides* (Willd.) Vatke, which mainly parasitizes the legume crop cowpea, is widely distributed in Africa. The most common *Orobanche* species, *viz*. *Orobanche crenata* Forsk. and *Orobanche aegyptiaca* Pers., predominantly occur in the Mediterranean area, eastern Europe and in the Middle East. *Orobanche crenata* has a rather narrow host-range and faba bean is the most seriously affected, whereas *Orobanche aegyptiaca* has a wide host-range and parasitizes on several dicotyledonous crops such as tomato, tobacco and sunflower.

Orobanche species, like other Orobanchaceae, are obligate root parasites and depend entirely on their hosts for all nutritional requirements. They are devoid of leaves and lack the ability to photosynthesize due to extensive deletion and rearrangement of the chloroplast genome[5]. *Striga and Alectra* develop green leaves and posses intact chloroplast genomes[6]. Therefore,

they were first thought to supply their own needs for organic matter and to rely upon their hosts only for water and minerals. However, it was found that they exhibit only a low rate of photosynthesis[7] and there is a substantial movement of organic matter to these parasites from their host[8]. The life cycle of the parasitic weeds *Striga* and *Orobanche* is extremely well adapted to their host plants. This life cycle exhibits two main phases: independent and parasitic. The independent phase begins with germination and lasts only a few days until the haustorium is established. This phase is entirely based on the consumption of material stored in the seeds. The parasitic life phase starts as soon as a haustorium has developed. At this point the parasite becomes dependent on nutrients derived from the host. The haustorium is an organ used as a physiological bridge between the vascular system of the host and the parasite. Subsequent to the establishment of the conductive connection, the parasite develops a shoot that emerges from the soil, flowers and sets seeds. Each *Striga* and *Orobanche* plant produces thousands of seeds. These seeds are extremely small, between 200 and 400 μm and each weighing ca. 15-25 μg. The ability of these seeds to remain viable and dormant for many years results in a dramatic increase of the parasite seedbank[2]. The level of infestation may become so great, that normal cereal production is impossible and farmers abandon these fields in search of less infested areas. In West Africa, it was estimated that about 40 million hectares in cereal production were heavily infested by *Striga* spp., while nearly 70 million hectares have moderate levels of infestation.[9] The Food and Agriculture Organization of the United Nations (FAO) estimated that annual yield losses due to *Striga* spp. in the savannah regions alone account for US $ 7 billion and are detrimental to the lives of over 100 million African people.[10] *Orobanche* damage to tobacco and sunflower is increasing steadily and has become a serious treat in Eastern Europe, and also in France, Spain and Germany.[11]

1.2 Naturally Occurring Germination Stimulants

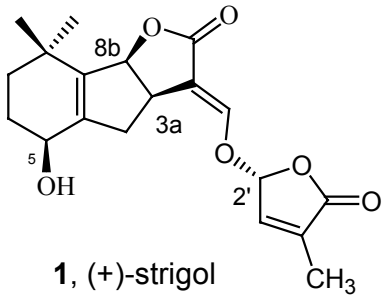
Strigolactones

Strigolactones serve as allelochemicals,[12] a class of molecules that is produced by plants and which affects the growth and development of neighbouring plants. Strigolactones are produced in the rhizosphere of host plants and some non-host plants that induce germination of seeds of parasitic weeds. Thus far, four germination stimulants (strigolactones) have been isolated *viz*. (+)-strigol (**1**),[13,22] (+)-sorgolactone (**2**),[14] alectrol (**3**)[15,16] and (+)-orobanchol (**4**)[16] (figure 1). The first naturally occurring germination stimulant identified is (+)-strigol (**1**)**.**[13] This compound was isolated in 1966 from the root exudates of cotton (*Gossypium hirsutum* L.) which, however, is not a host for *Striga* and *Orobanche*. As such, its

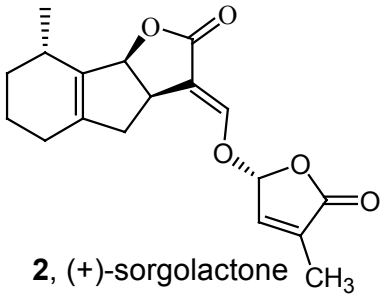
significance in the true host-parasite interaction was uncertain for a long time. The relative configuration of (+)-strigol was determined in 1972,[17] while the absolute stereochemistry was established unambiguously in 1985 by means of an X-ray diffraction analysis.[18] It was not until 1992 that (+)-sorgolactone (**2**)[14] an analogue of strigol, was isolated from sorghum roots (*Sorghum bicolor* L. Moench). The proposed structure for this stimulant was confirmed by its total synthesis accomplished in 1998 by the Nijmegen research group[19] and by Mori *et al.*[20] Soon after the isolation of sorgolactone, Schildknecht *et al*[15] reported the isolation of a germination stimulant alectrol (**3**). A structure has been proposed for alectrol (figure 1), although recently it was shown by the synthesis of the proposed structure that the spectral data are not in agreement with those of the natural stimulant.[21] (+)-Strigol itself was shown to be the major germination stimulant for *Striga* produced by maize (*Zea mays* L.) and proso millet (*Panicum miliaceum* L.).[22] In addition, it has been shown that root exudates of *Striga* hosts generally contain a mixture of the strigolactones, although in different ratios.[22]

Orobanchol (**4**) is the first stimulant isolated from the *Orobanche* host red clover (*Trifolium pratense*).[16] As only minute amounts of stimulant could be obtained from its natural source, the structure elucidation was mainly based on GC-MS data.[23] The synthesis of this stimulant was recently published by Mori *et al*.[23]

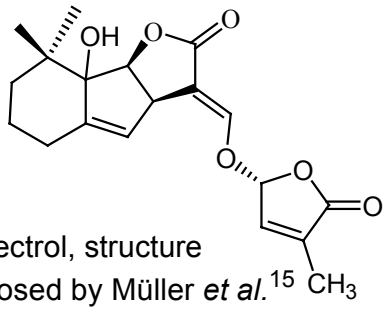
Figure 1



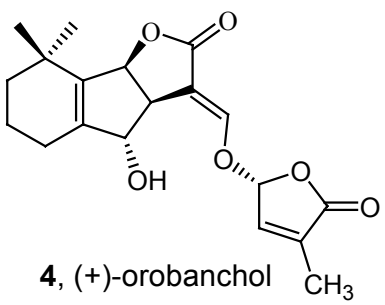
1, (+)-strigol



2, (+)-sorgolactone



3, alectrol, structure proposed by Müller *et al.*[15]



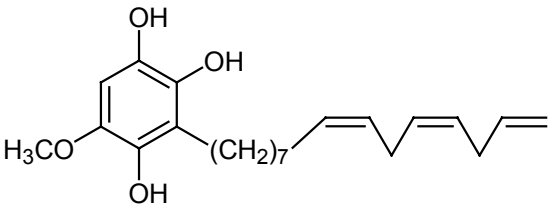
4, (+)-orobanchol

Other germination stimulants

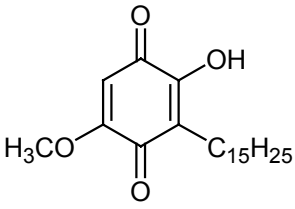
Several reports have appeared claiming that seed germination of *Striga* and *Orobanche* is induced by other natural compounds including sesquiterpene lactones (which are not strigolactones), cytokinins, auxins, giberellins, cotylenins, fusicoccins and jasmonates.[24,25,26,27,28] It is important to note that, in general, these other germination stimulants exert their effect at much higher concentrations than the strigolactones and thus have a much less specific mode of action. Moreover, the germination assays of these compounds are not always unambiguous.[29] Another germination stimulant is the plant hormone ethene. The ethene gas is able to stimulate the germination of *S. asiatica* and *S. hermonthica.*[30,31] In fact, ethene gas has been used to eradicate *S. asiatica* in the USA.[32] For this purpose, ethene was injected into the soil under high pressure using highly specialized equipment.

Although effective, injection of ethene gas is potentially dangerous, very costly, and as such, generally unsuitable in Africa. However, a recent report has shown that under laboratory conditions, the bacterium *Pseudomonas syringae* pathovar glycinea synthesizes relatively large amounts of ethene.[33] These bacteria were found to be consistently better stimulators of germination of *Striga* spp. seeds, when compared with ethene gas or root pieces of a *Vigna unguiculata* cultivar (known to stimulate germination of parasite seeds). Furthermore, *Ps. syringae* were as effective in germinating *S. aspera* and *S. gesnerioides* seeds as the synthetic germination stimulant GR24. These results suggest that these bacteria may provide a practical means of biological control of *Striga spp*. in places where high-pressure injection is not applicable.

In 1986, it was claimed that dihydrosorgoleone (SxSg) (**5**) was the actual germination stimulant exuded by sorghum roots.[34] However, the compound was found to be highly unstable and readily oxidized to the corresponding inactive quinone, sorgoleone (**6**), and it is this compound that constitutes more than 90% of sorghum root exudates.[35,36] Moreover, sorgoleone (**6**) exhibits no germination activity. Notably, neither (**5**) nor (**6**) was ever detected in the root exudates of maize, which is highly susceptible to *Striga.*[37] A detailed discussion on the possible role of SxSg in the inception of *Striga* seed germination has been reported recently.[38]



5, dihydrosorgoleone (SXSg)



6, sorgoleone

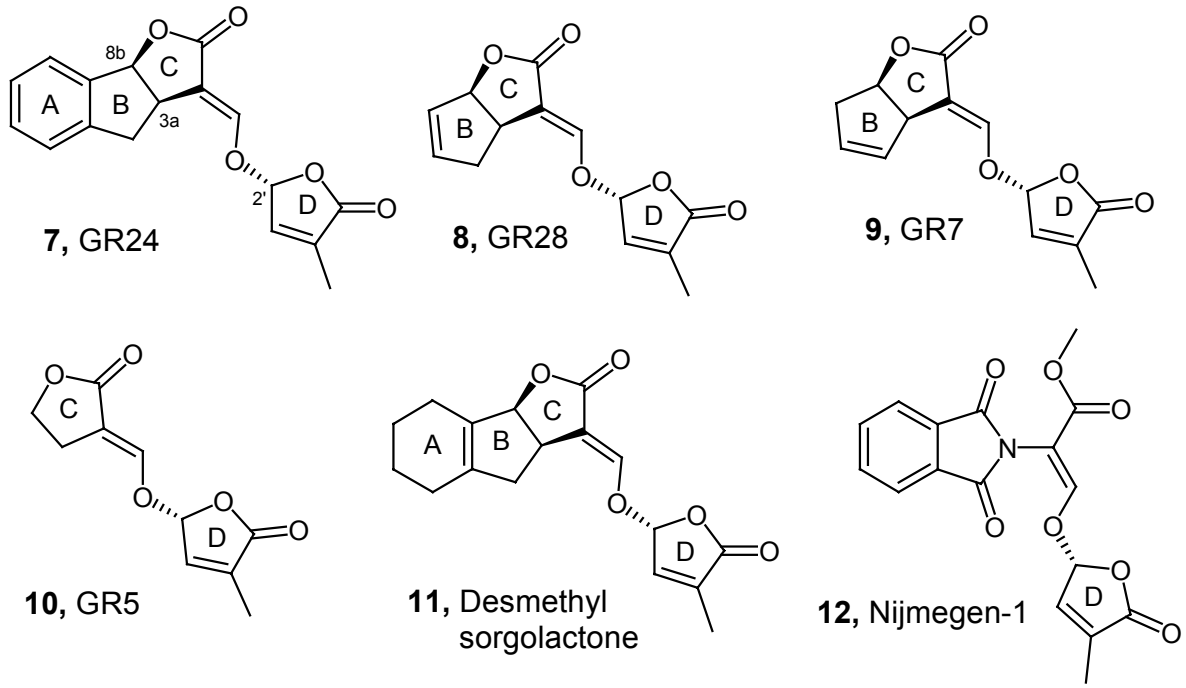
1.3 Strigolactone analogues and their biological activity

Since the elucidation of the structure of (+)-strigol,[17] much attention has been given to the synthesis of strigolactone analogues, in order to obtain stimulants with simplified structures that might be used to induce suicidal germination of seeds of parasitic weeds.[39-44] The term 'suicidal germination' represents a selective method for the control of seeds of parasitic weeds involving seed germination in the absence of the host plant and which results in starvation of the seedling after a short time. Thus, introducing a stimulant into the soil to induce germination of seeds of the parasitic weeds before planting the desired crop. Several strigolactone analogues, active,[39,40,41,42,46,48] and inactive[43,44,45] have been prepared. The active strigolactone analogues were prepared by modifications at the A/B rings of strigolactones, whereas the CD part remained unaffected. This is in contrast to the inactive analogues which were prepared by modification of strigolactone at the CD part of the molecule. These analogues exihibit no stimulatory activity in the seeds of *Striga* and *Orobanche*. Structure activity studies have led to the identification of the bioactiphore in strigolactone. It was demonstrated that the CD-part of the stimulant molecule is primarily responsible for the activity.[42,50] In this section, only the most common and active synthetic analogues will be discussed (figure 2).

The first contribution to the synthesis of strigolactone analogues came from the group of Johnson,[39,46] who prepared a series of so-called GR-compounds, *viz*. GR24 (**7**), GR28 (**8**), GR7 (**9**), GR5 (**10**) (figure 2). Among these analogues, GR24 (**7**) is one of the most potent synthetic germination stimulants.[39,40,47] This stimulant is used world wide in parasitic weed research to induce germination and as a standard for comparing the activity of new germination agents. Multigram scale production of GR24 is not (yet) economically feasible, which prevents its use as a suicidal germination agent. Nijmegen-1 (**12**) which has a much more simplified structure, was designed to contain the essential structural features required for bioactivity.[42] This stimulant is derived from *N*-Phthaloyl glycine, which can be readily

prepared in multigram quantities and is an attractive candidate for parasitic weed control by the suicidal germination approach.

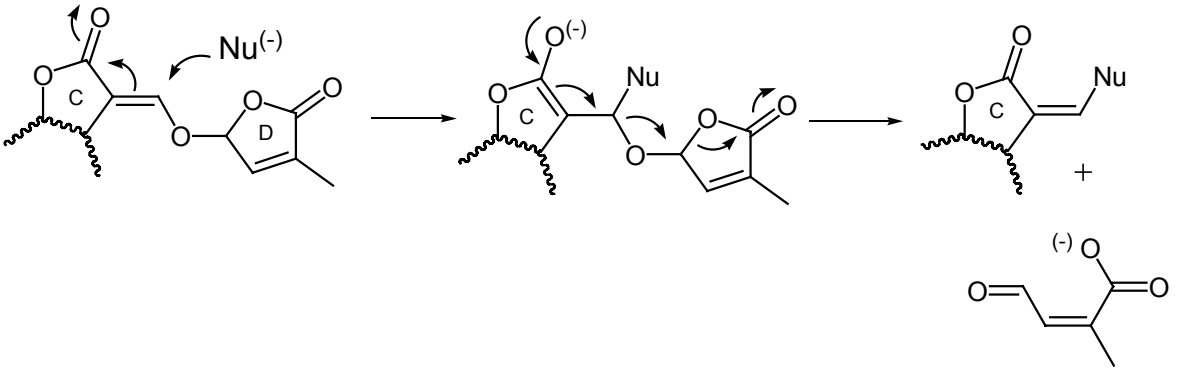
Figure 2



The preparation of all the possible stereoisomers of GR24 (**7**)[40], GR7 (**9**),[48,49] desmethyl sorgolactone (DMSL) (**11**)[41] and Nijmegen-1 (**12**)[42] in enantiopure form has been reported by the Nijmegen research group. In chapter 5 of this thesis, an alternative asymmetric synthesis of GR24 (**7**), GR7 (**9**) and Nijmegen-1 (**12**) is presented. Structure-activity relationships of these stimulants revealed that *i.* the synthetic analogues are active at low concentrations. It was reported that racemic GR24 (**7**) is almost as active as the single isomer of the natural stimulant (+)-sorgolactone (**2**).[47] The ED_{50} values (half-maximum percentage of germination) reported for GR24 were in the order 10^{-8} to 10^{-9} mol/L and for (+)-sorgolactone 10^{-10} mol/L. Nijmegen-1 is considerably less active than GR24 in stimulating the germination of *Striga* seeds. Its E_D values are up to 3 order of magnitude higher than those of GR24 and sorgolactone.[47] *ii*. dose–response curves of GR24 and Nijmegen-1 have a bell- shape.[47] Those curves define the optimal concentrations at which the stimulants are active. In the case of GR24, germination generally starts at a concentration of 10^{-9} mol/L and reaches a maximum value at a concentration of 10^{-7}-10^{-8} mol/L. At a concentration below 10^{-10} mol/L no germination activity was observed. At a concentration higher than 10^{-4} mol/L, germination

often decreases considerably. *iii*. the absolute configuration of the CD-part of the stimulant, natural or synthetic, is of great importance for the germination activity.[41,50] For example, the influence of the stereochemistry on the bioactivity of desmethyl sorgolactone (DMSL) (**11**) was investigated for the seeds of *Striga* and *Orobanche*[41]. This stimulant contains two stereogenic centers, *viz.* C3a/C8b and C2', giving rise to four stereoisomers. A significant difference in the stimulatory activity between the four stereoisomers was observed. The isomer having the "natural" absolute stereochemistry at the CD part, *viz.* 3a(R),8b(S),2'(R), was considerably more active than its optical antipode. The difference in activity amounts at least to a factor of 100. Similar results were obtained for GR24[40]. *iv*. the bioactiphore being the part of stimulant molecule that is responsible for biological activity, can be identified. It appeared that the CD-part of the molecule, including the enol ether linkage, is the minimum structural requirement for biological activity.[42,51] An analysis of the bioassays of synthetic analogues and natural stimulants has led to the hypothesis that the induction of *Striga* or *Orobanche* seeds germination proceeds via a receptor-mediated mechanism.[38,51] Further elaboration on this hypothesis is presented in chapters 5 and 6 of this thesis.

Scheme 1

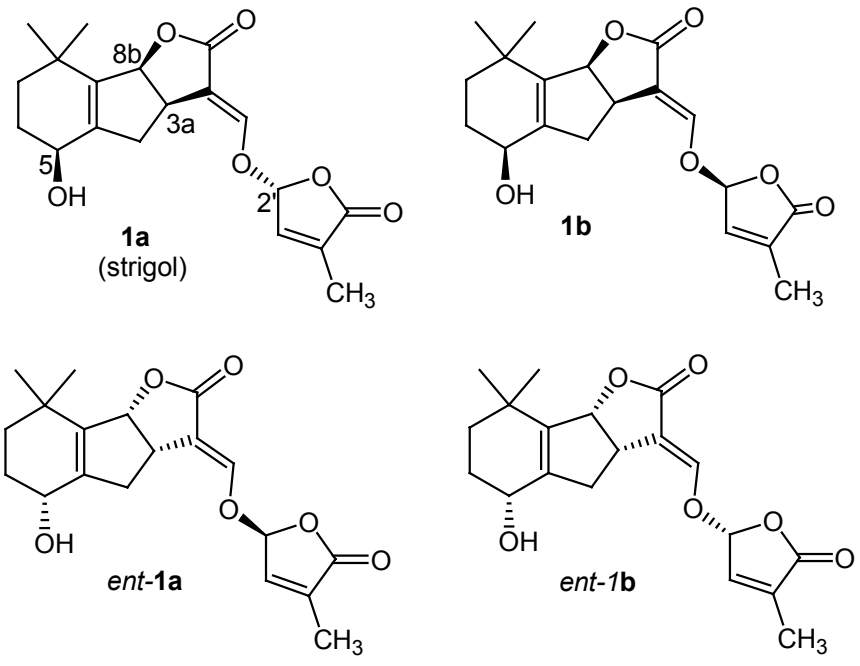


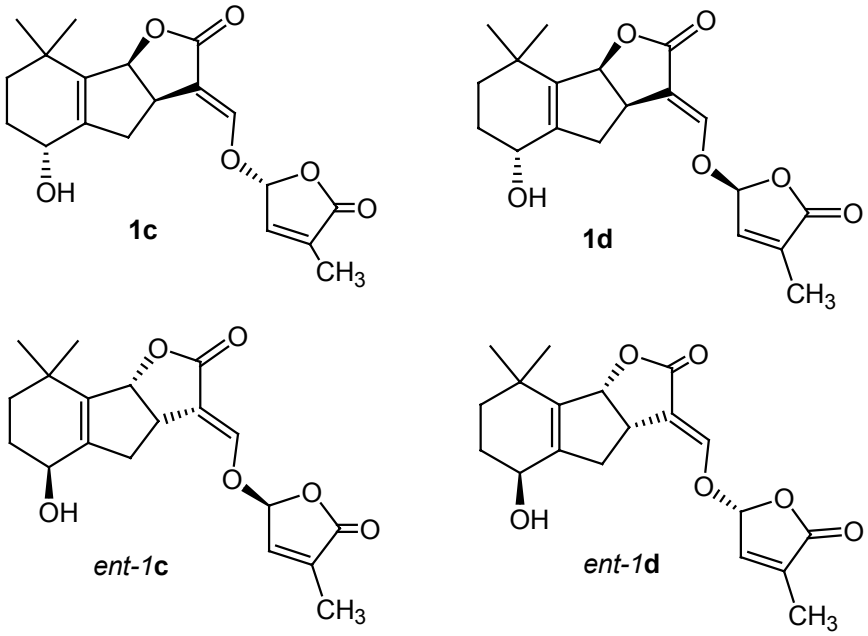
Magnus and Zwanenburg have proposed a tentative molecular mechanism for the initial stages of the germination of parasitic weeds seeds[51] (Scheme 1). This receptor-mediated mechanism involves addition of a nucleophilic species, available at the receptor site, in a Michael fashion and subsequent elimination of the D-ring. The result is that the ABC-part of the stimulant is covalently bound to the receptor. This chemical change may be responsible for initiating germination. Support for this mechanism was provided by studying several synthetic modifications at the CD-part of the stimulant molecule, e.g. replacement of the vinyl ether linkage by a single bond resulted in complete loss of activity. Substitution of the vinyl ether oxygen by a carbon atom also led to an inactive molecule. In this carba-analogue the D-ring can no longer function as a leaving group.[44]

1.4 Approaches to the synthesis of the strigol ABC-part

The naturally occurring germination stimulant (+)-strigol (**1**) has received much attention from synthetic chemists. Following the isolation of (+)-strigol in 1966, various attempts were made to prove or revise tentatively assigned structures as well as to obtain sufficient material for biological studies. The natural material can be obtained in only minute quantities after tedious isolation and purification which has impeded these biological studies considerably.[13] Therefore, it was desirable to devise a practical, chemical synthesis of this molecule to make it available in quantities sufficient for detailed investigations. Strigol (**1**) contains four stereogenic centers, *viz.* C3a/C8b, C5 and C2'. However, in view of the high strain between the B and C-rings, only a *cis* relationship can exist between these rings, therefore C3a/C8b can be regarded as one stereogenic center, leaving eight stereoisomers for strigol (figure 3). Several total and partial syntheses of strigol have been reported.[52,53,54,55,56,57,58,59,60]

Figure 3

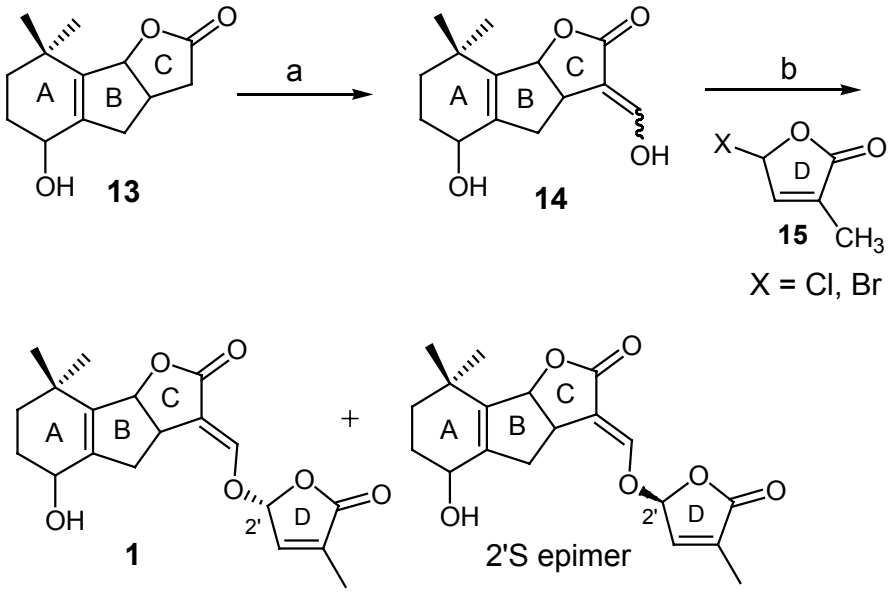




Most of the synthetic routes described for strigol, involve consecutive A+B+C+D ring formation. In all these syntheses, the final step comprises the coupling of formylated tricyclic lactone ABC-part **14** to the butenolide D-ring precursor **15**. The resulting products are exclusively formed with the desired *E*-geometry of the enol double bond (Scheme 2). The two racemic diastereoisomers of **14** couple with the D-ring precursor **15** to give two racemic diastereoisomers of strigol and its 2'*S*-epimer.

The synthesis of the tricyclic building block **13** has been achieved following a number of conceptually different approaches which will be outlined in this section. The first synthesis of the four racemic stereoisomers of (±)-strigol was reported by MacAlpine in 1974.[52] The starting material for the synthesis of the ABC-fragments *rac*-**13a** and *rac*-**13b** is the readily available 2,2-dimethylcyclohexanone (**16**) which was condensed with either the Grignard or the lithio-derivative of 3-tetrahydropyran-2-yloxypropyne (**17**) to produce, after acidic hydrolysis, the diol **18**. For the preparation of **19**, which is the AB fragment of strigol, the diol **18** was treated with phosphorus pentoxide in methanesulfonic acid. This step involves a Rupe rearrangement with a subsequent Nazarov-type conrotatory electrocyclisation (Scheme 3).

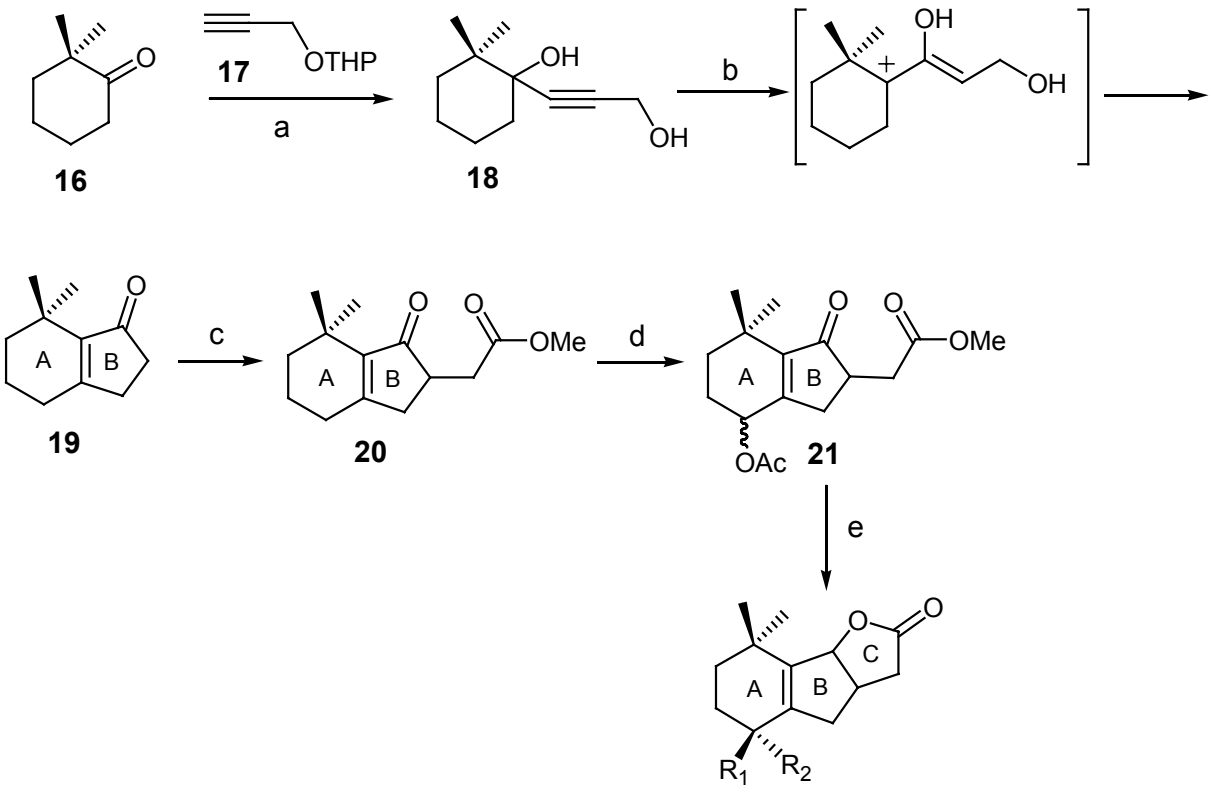
Scheme 2



Reagents and conditions: a. NaH, HCOOEt, Et_2O; b. K_2CO_3, bromobutenolide, DMF.

The introduction of the C-ring to the AB-fragment **19** involved activation of the ketone at the α–position by condensation with diethyl oxalate. Then reaction with methyl bromoacetate and subsequent removal of the oxalyl group gave the crystalline ester **20**. A crucial step in the sequence to the appropriately functionalized ABC-part of strigol is the introduction of the hydroxy group to the A-ring. MacAlpine used a highly regioselective bromination of the ester **20** employing *N*-bromosuccinimide (NBS) in carbon tetrachloride, followed by substitution of the thus obtained allylic bromide with silver acetate to give the diester **21** as a mixture of two diastereoisomers. Subsequent hydrolysis of the diesters, followed by reduction of the keto group and concomitant lactonization gave two pairs of racemic ABC-diastereoisomers, rac. **13a** and rac. **13b**, that could be separated by crystallisation. The key intermediate in the synthesis of ABC-fragment **13** from citral (**22**), as has been reported by Sih[53], is compound **26** (Scheme 4). Citral was cyclized with sulfuric acid at –20°C to afford a mixture of α-cyclocitral (**23a**) and β-cyclocitral (**23b**) in a ratio of 10:1. Since α-cyclocitral is readily converted into its isomer **23b** by base treatment, either **23a** or **23b** could be produced in a good yield from citral, and both were used in separate routes to **13**. Epoxidation of **23a** followed by opening of the epoxide with pyrrolidine and subsequent oxidation of the resulting hydroxy aldehyde **24** gave the acid **25** in a good yield.

Scheme 3

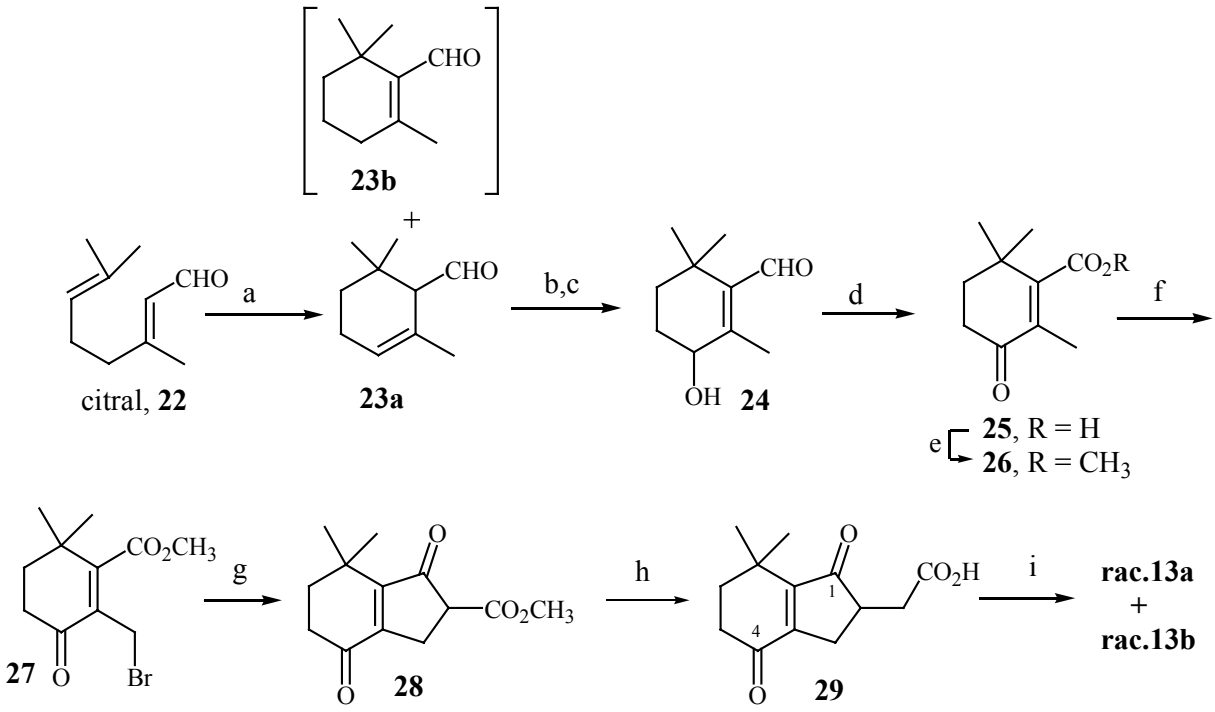


rac.13a R_1:OH R_2:H,crystalline
rac.13b R_1:H R_2:OH,oil

Reagents and conditions: a. *i* EtMgBr, reflux, *ii* H_2SO_4, MeOH, b.P_2O_5, $MeSO_3H$, -15°C, c. *i* diethyl oxalate, NaH, methyl bromoacetate, *ii* NaOMe, MeOH, d. *i* NBS, AIBN, CCl_4, *ii* AgOAc, HOAc, e. *i* NaOH, HCl (pH 4) *ii*. DIBAH, toluene, -70°C.

Esterification of **25** with methyl iodide and potassium carbonate afforded the corresponding ester **26**. For the construction of the five-membered ring, the keto ester **26** was brominated with *N*-bromosuccinimide (NBS) followed by treatment with excess sodium dimethyl malonate in methanol, resulting in alkylation and cyclization to give the β-keto ester **28**. In a manner similar to that of MacAlpine for the construction of the C-ring, alkylation of the ester **28** with methyl bromoacetate followed by hydrolysis and decarboxylation under acidic conditions, gave the diketo acid **29**. A suitable reducing agent to reduce both ketones at C1 and C4 was diisobutylaluminum hydride (DIBAH) in dichloromethane. The *in situ* formed diol yielded a mixture of the lactones rac.**13** and rac.**13b**.

Scheme 4

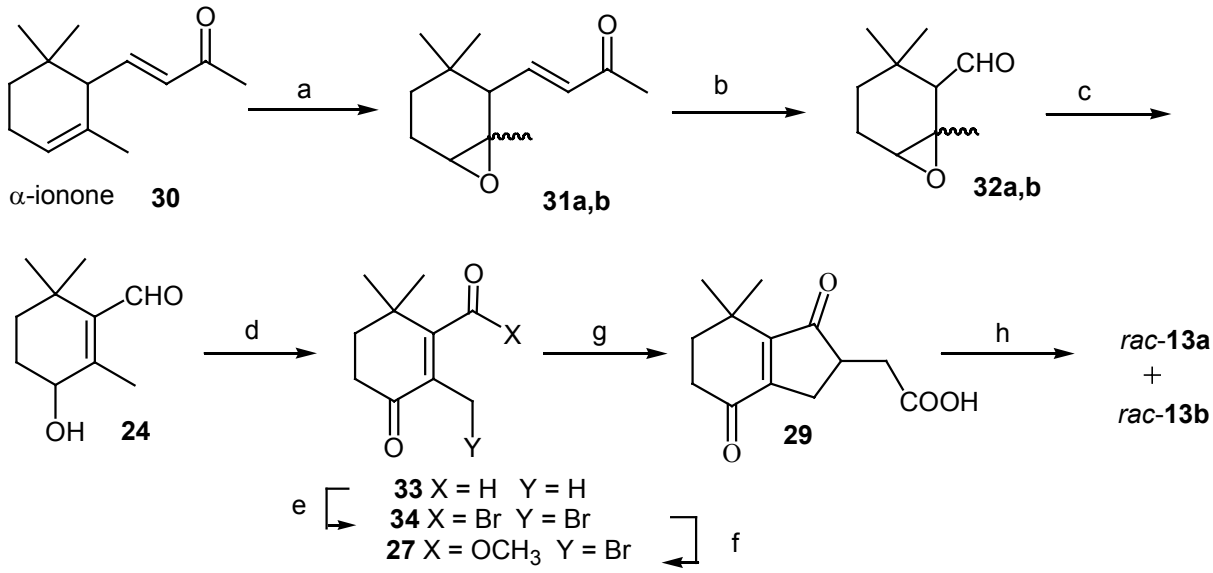


Reagents and conditions: a. *i* $PhNH_2$, *ii* 95% H_2SO_4 –20°C, b. *m*CPBA, c. pyrrolidine. d.CrO_3, H_2SO_4, e. MeI, K_2CO_3, f. NBS, CCl_4, g. *i* $Na^+CH(CO_2CH_3)_2$, *ii* HOAc, h. *i* methyl bromoacetate, K_2CO_3, *ii* HOAc, HCl, H_2O,
i. DIBAH, CH_2Cl_2, -70°C.

Several years later Brooks and co-workers[54] published an improved total synthesis of (±)-strigol and (±)-2'-epistrigol. This synthesis is applicable on a multigram scale with a minimum of purification steps. For the construction of the ABC-lactones **13a** and **13b**, commercially available α-ionone (**30**) was chosen as the starting material as it contained the required carbon framework and the appropriate functionalities for elaboration to an A-ring intermediate (Scheme 5). Epoxidation of **30** with peracetic acid provided a mixture of the isomeric epoxides **31a** and **31b** in a ratio of 9:1 in a good yield. The enone function was removed by ozonolysis and subsequent reduction with zinc in acetic acid gave the aldehydes **32a** and **32b**. Epoxide opening with pyrrolidine followed by selective oxidation of the thus obtained hydroxy aldehyde **24** using Jones' reagent gave the corresponding keto aldehyde **33**. This compound could be converted directly into the bromomethyl ester **27** by a one-pot reaction with NBS in carbon tetrachloride using a sun lamp. The intermediate dibromide **34** was not isolated but treated with excess methanol to give **27**. For the formation of B-ring unit,

the method reported by Sih *et al*[53] was used. Treatment of **27** with dimethyl malonate and sodium hydride in THF gave the keto ester **29**. Ring closure to the C-ring was achieved as described by Sih *et al*[53].

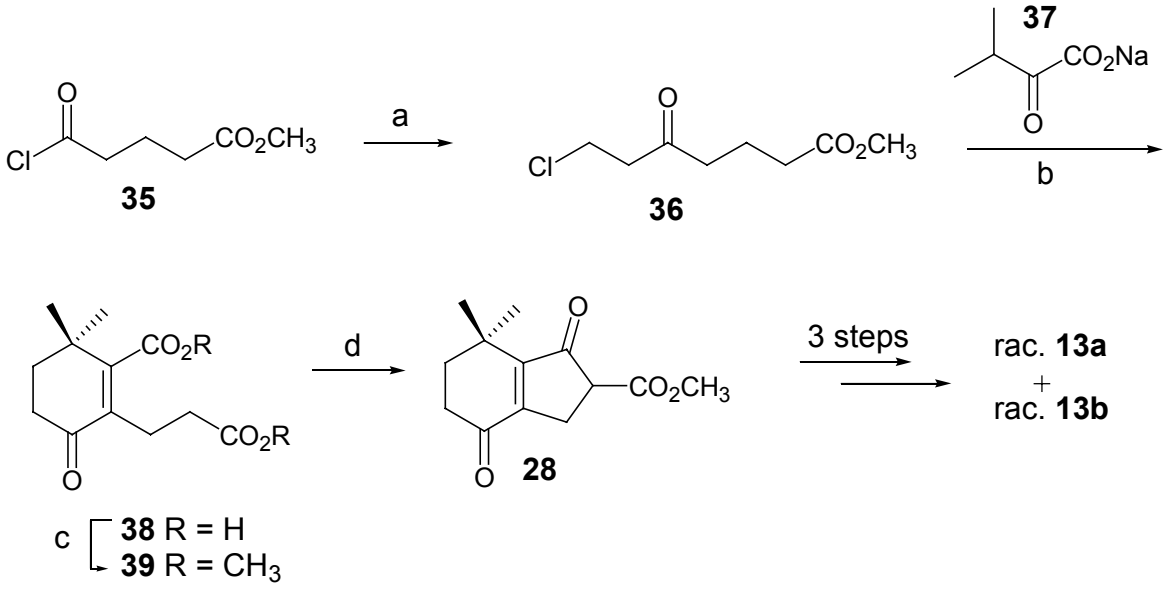
Scheme 5



Reagents and conditions: a. *i* CH_3CO_3H, CH_3CO_2H, 0°C, b. *i* O_3, CH_3OH, -78°C *ii* Zn, AcOH, 30°C to 25°C, c. pyrrolidine, ether, d. CrO_3, H_2SO_4, e. *i* NBS, CCl_4, 70°C, hν 2-3 hrs f. CH_3OH, 0°C, 6 hrs g. *i* NaH, $CH_2(CO_2CH_3)_2$, THF *ii* methyl bromoacetate, h. HOAc, HCl, 10 °C, g. $CeCl_3$, $NaBH_4$, 0°C.

Several total and partial syntheses of strigol have been reported by Welzel *et al*[56,57] in the 90's. The syntheses of **13** in a racemic as well as in enantiopure form were accomplished. The synthesis of enantiopure **13a** using the chiral pool approach is elaborated in section 1.7 of this chapter. The Welzel synthesis of rac. **13**[57] is a slightly modified procedure reported earlier by Dolby.[60] The key intermediate is the keto ester **39** which was obtained by condensation of **36** with sodium 3-methyl-2-oxobutanolate **37** in aqueous base to give cyclization product **38** in 53% yield (Scheme 6). Esterification of **38** gave **39** which in a Dieckmann condensation gave diketone **28**. This route to the hydrindan portion of strigol is much shorter than those previously reported and the yields are good. The conversion of **28** into **13** was performed as outlined in scheme 4.

Scheme 6



Reagents and conditions: a. ethene, $AlCl_3$, CH_2Cl_2, b. aq. KOH (1.5N), 100°C and then acidification with 5% HCl, c. DBU, MeI, d. $NaOCH_3$, CH_3OH, reflux followed by acidification with HOAc.

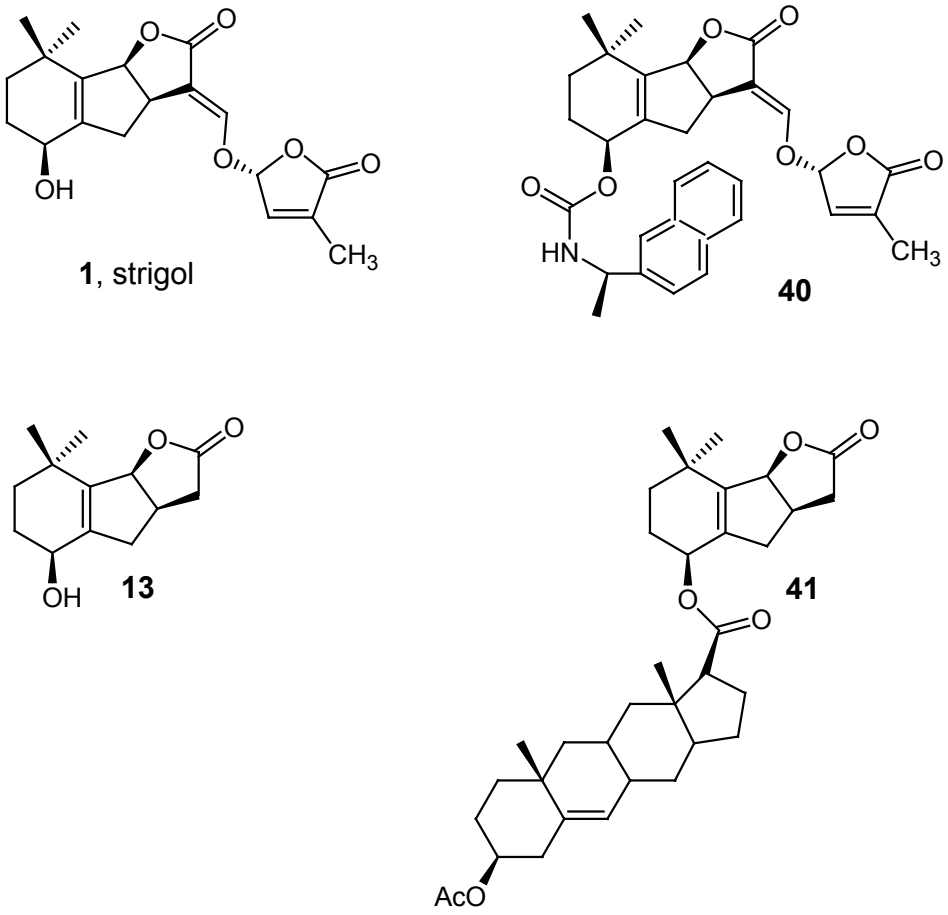
1.5 Asymmetric synthesis of strigolactones

Over the years extensive efforts were reported to prepare natural and synthetic stimulants in enantiopure form. The aim was to gain more insight in the structure-activity relationships of these stimulants. The principal strategies reported hitherto for the synthesis of enantiopure strigolactones are: *i* resolution of the racemic stimulant in the final stage, *ii* coupling of the enantiopure ABC fragment with the racemic D-ring precursor and subsequent chromatographic separation of the thus obtained diastereomeric mixture, *iii* coupling of the racemic ABC fragment with enantiopure D-ring precursor and subsequent separation of the resulting diastereisomers, *iv*. using the chiral-pool approach for the preparation of enantiopure ABC-part.

Methodology i. Resolution of the racemic stimulant at the final stage by covalent coupling with a suitable resolving agent was reported by Brooks *et al*.[18] The racemic (±)-strigol was resolved via the corresponding N-[(*R*)-1-(1-naphthyl)ethyl]carbamate **40** (figure 3), whose structure was solved by an X-ray crystallographic analysis. Separation of the thus obtained diastereomeric mixture and subsequent cleavage of the carbamates under mild conditions, gave the enantiopure (+)-strigol and (-)-strigol in a good yield. A different approach was reported by Hauck *et al*. who obtained enantiopure (+)-strigol by chromatographic separation

of the racemic (±)- strigol on a chiral column of cellulose triacetate.[61] In a similar fashion, the separation of (±)-strigol was achieved on a chiral column of cellulose carbamate, as will be described in chapter 2. In this manner, all the eight stereoisomers of strigol could be obtained as single compounds.[59] This method was also employed to obtain some synthetic analogues, *viz.* deshydroxy strigol[62] and methyl substituted GR24,[63] in enantiopure form. It should be mentioned that separation of enantiomers on a chiral preparative HPLC column is convenient when a small amount of material is required for biological testing.

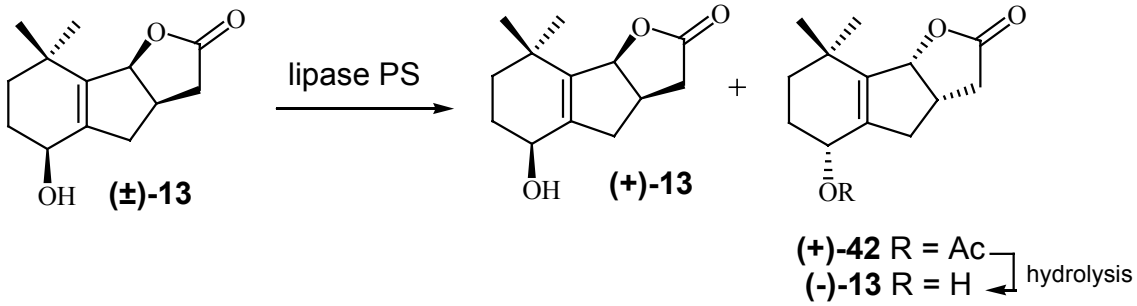
Figure 3



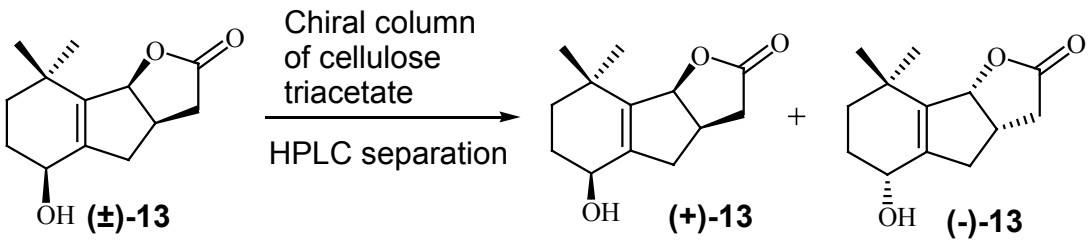
Methodology ii. Coupling of the enantiopure ABC fragment, with the racemic D-ring precursor and subsequent chromatographic separation of the thus obtained diastereomeric mixture was reported by Sih *et al*[53]. The enantiopure ABC-hydroxy lactone (+)-**13** was obtained by coupling with a suitable resolving agent to give the corresponding ester **41** (Figure 3). Separation of the thus obtained diastereomeric mixture and subsequent cleavage of the ester, gave the enantiopure (+)-**13a** and (-)-**13a**. Coupling of optically pure ABC-part (+)-

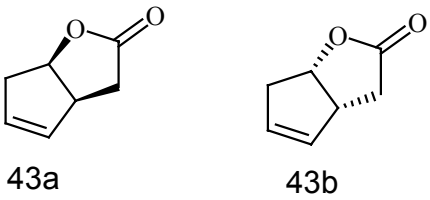
13a with the racemic bromobutenolide D-ring precursor rac.**15** gave a mixture of (+)-strigol and the corresponding C2'-epimer, (+)-2-epistrigol, that could be separated by column chromatography to give both diastereomers in enantiopure form. Alternatively, Mori *et al*[58] used an enzymatic acetylation of (±)**-13** with vinyl acetate in the presence of lipase AK (Scheme 7), whereas Welzel and co-workers resolved (±)**-13** by chromatographic separation using a HPLC chiral column of cellulose triacetate[57] (Scheme 8). Chromatographic separation of the ABC-fragment of GR24 was also achieved using cellulose triacetate (CTA) as the chiral stationary phase.[40] Mangnus *et al*. have applied this methodology for the synthesis of GR7 using commercially available lactones **43a** and **43b**, also known as Corey's lactones.[48]

Scheme 7



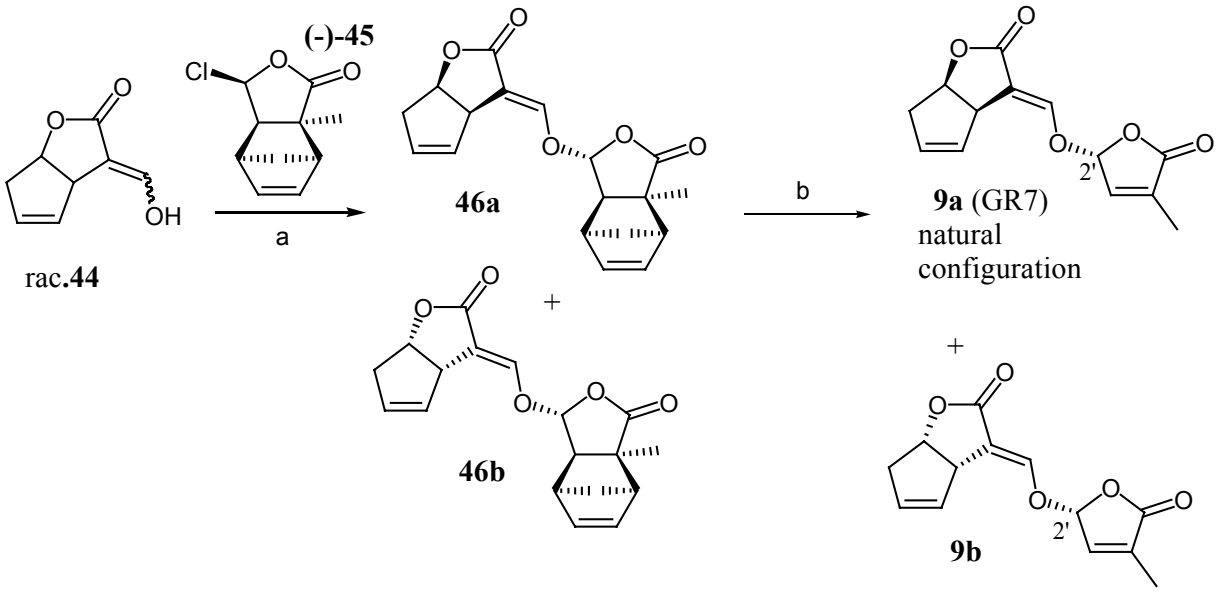
Scheme 8





Methodology iii. The coupling of racemic ABC-fragments with enantiopure D-ring precursor was successfully developed by Thuring *et al*. This method involves the control of stereochemistry at C2' using the enantiopure D-ring synthon **(-)-45**[49,64] (Scheme 9). Coupling of ABC-precursor rac**. 44** with precursor **(-)-45** proceeds with complete exo-selectiveity with respect to the norbornen bridge. This reaction gave a mixture of two diastereoisomers, namely, **46a** and **46b**. Separation of these diastereoisomers and a subsequent retro Diels-Alder reaction gave optically pure strigolactones **9a** and **9b**. The first mentioned one is GR7 with the natural configuration at C2' and the stereogenic center at B/C ring junction. This methodology was successfully applied for the preparation of all eight stereoisomers of sorgolactone[19] and the synthetic stimulants GR7[49], GR24,[40] Nijmegen-1[42] and desmethylsorgolactone[41] in enantiopure form.

Scheme 9

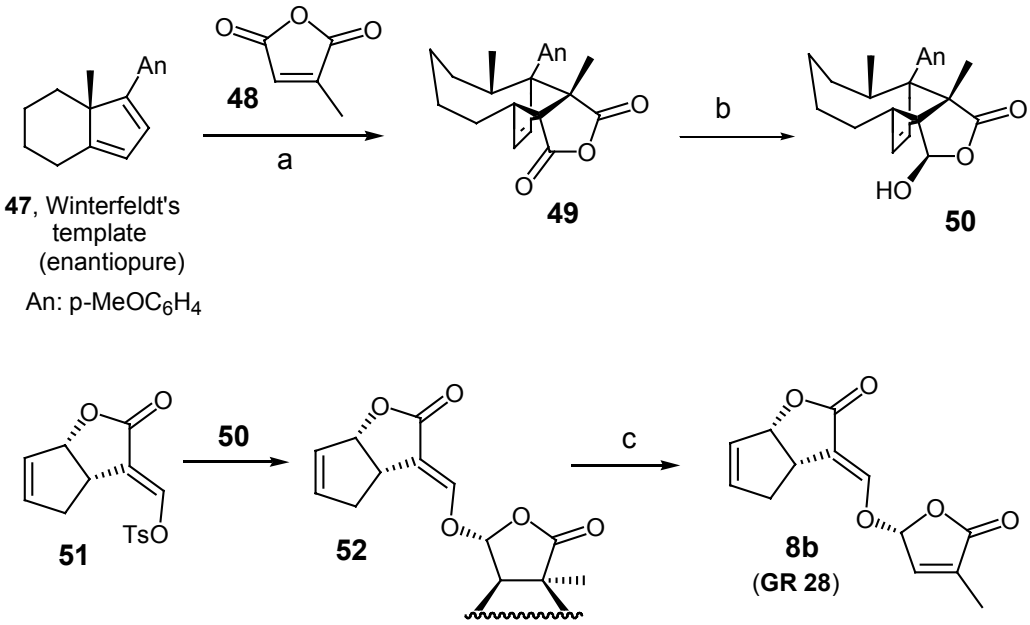


Reagents and conditions: a. KO^{t}Bu, DMF, 20h, separation of diastereoisomers. b. *o*-dichlorobenzene,180ºC.

Welzel *et al.* installed the correct stereochemistry at C2' in a highly selective manner using Winterfeldt's template as a chiral auxiliary (Scheme 10).[65,66] Reaction of template **47** with citraconic anhydride at high pressure gave adduct **49** which was then reduced with $LiAl(OtBu)_3H$ to give **50**. This D-ring precursor was coupled with enol tosylate **51** to produce **52** which on subsequent flash vacuum thermolysis gave the desired enantiopure product **8b.** This is a diastereoisomer of GR28. Winterfeldt's auxiliary can be obtained from the chiral Hajos-Wiechert ketone,[67] of which only one enantiomer is readily accessible. Hence, Welzel's approach is restricted to the synthesis of **8b**, where as the Nijmegen approach[19,40,41,42] involving the use of **45** which is attainable in both enantiomeric forms, both D-ring epimers can be prepared.

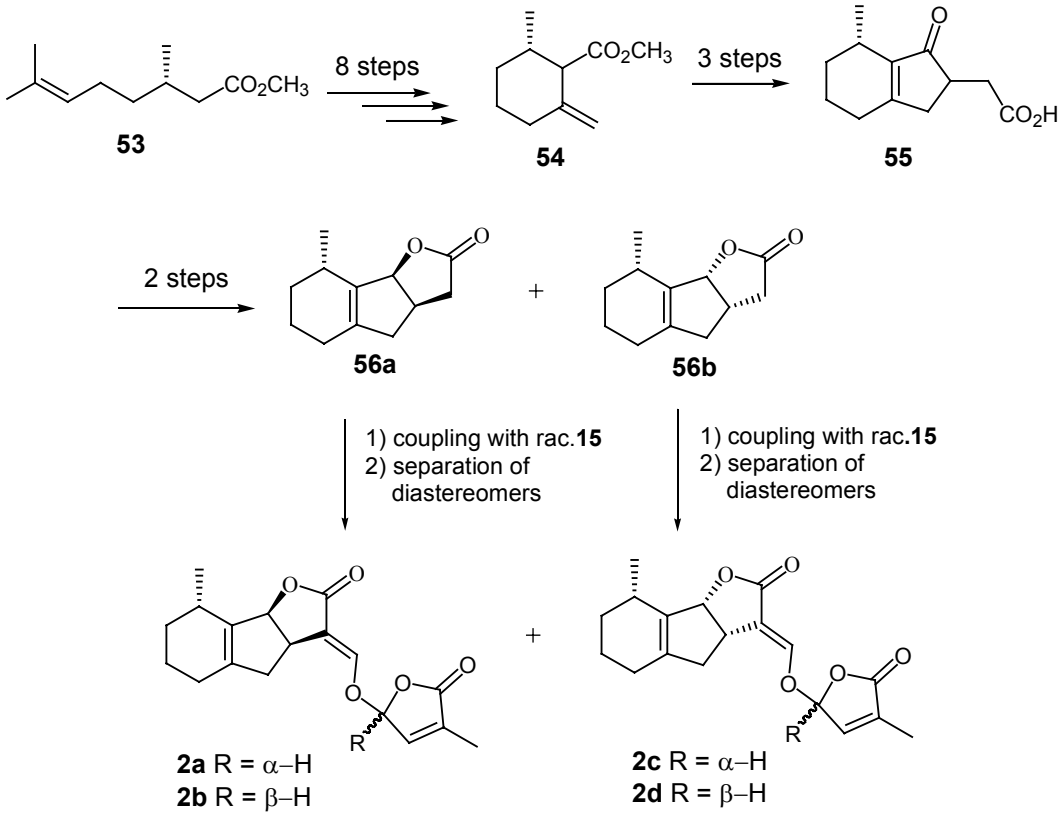
Methodology iv. The chiral pool approach for the synthesis of an enantiopure ABC fragment was reported by Mori *et al* for the synthesis of enantiopure sorgolactone stereoisomers.[20] The use of methyl (*S*)-(-)-citronellate (**53**) as a chiral starting material secured the correct stereochemistry at C8, and consequently only two ABC-lactones, *viz*. **56a** and **56b**, were obtained, both in enantiopure form (Scheme 11). The preparation of **56** from **53** required several steps, whereby **54** and **55** are intermediates. Coupling of **56a** and **56b,** respectively, with racemic bromobutenolide (rac. **15)**, followed by chromatographic separation, furnished the naturally occurring sorgolactone **2a** and three of its stereoisomers *viz* **2b**, **2c** and **2d**, all having the *S* configuration at C8. Welzel and co-workers reported the preparation of enantiopure **(+)-13** using the chiral pool approach.[68] The (*S*)-(-)-malic acid derivative **57** was transferred in a number of steps into synthon **58**, which contains the necessary functionalities to allow the elaboration to the ABC-tricyclic system (Scheme 12). Coupling of **58** with the A-ring synthon **59** and subsequent functional group manipulation, followed by an intramolecular Wittig reaction furnished dienoic acid **62**. The oxidative cyclization of **62** to give **(+)-13a** could be performed stereoselectively using hydrogen peroxide in the presence of a catalytic amount of diphenyldiselenide.

Scheme 10

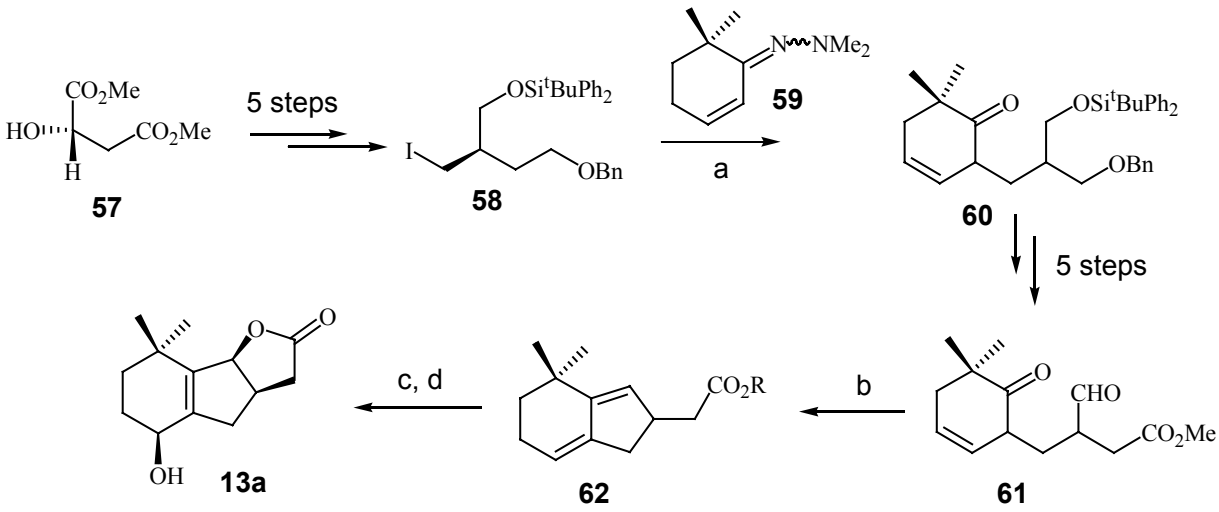


Reagents and conditions: a. 10 kbar, 72h, b. $Li(OtBu)_3AlH$, THF, c. FVT.

Scheme 11



Scheme 12



*Reagents and conditions: :*a. *i* **59** (1.2 equiv.) + LDA (1.4 equiv.) in THF, 1h, 0°C, *ii* at -78°C + **58**, -78°C-20 °C (5h), work-up (ether), *iii* + p-TsOH (1.5 equiv.) in wet ether, 5h, 20 °C, (iv) work-up. b. *i* Zn-Cu (2.36g) + $TiCl_3$ (11 equiv.) in DMF, 1h reflux, (ii) dropwise addition (97h) of **61** in DME at reflux temp.: 52%. c. *i* in THF + 0.15M aqueous LiOH, 5h at 0 °C, (ii) Dowex 50 (iii) *m*-CPBA (2 equiv.) in ether, -78°C-20 °C (2h), d. diphenyl diselenide (0.01 equiv.) + 30% H_2O_2 (1.9 equiv.) in THF, 3.5h at 0 °C: 58%.

1.6 Aim and outline of the thesis

The aim of the research described in this thesis is three fold. First, the synthesis of all eight stereoisomers of strigol was undertaken. Thus far, not all of these isomers had prepared and accordingly, their biological activity was unknown. Secondly, a new coupling method for the ABC-fragment in strigolactones and a D-ring precursor was investigated with the objective of preparing enantiopure strigolactones in a different and hopefully simpler manner. Thirdly, the biological function of the germination stimulant, especially the interaction with the receptor protein in the seeds of the parasitic weeds, was studied. One of the aims was the isolation of the protein that is involved in the germination process.

In chapter 1 the background of the research is described. Chapter 2 deals with the synthesis and biological evaluation of all eight possible stereoisomers of the germination stimulant

strigol. In chapter 3 the synthesis of the germination stimulants (±)-orobanchol and (±)-strigol via an allylic rearrangement is described. Chapter 4 deals with an efficient enantioselective synthesis of some strigolactones using a palladium-catalyzed asymmetric coupling as the key step. Chapter 5 and 6 are devoted to the isolation and purification of the protein receptor, involved in the germination of the seeds of parasitic weeds. This thesis is concluded with summaries in English and Dutch.

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CHAPTER 2

Synthesis of All Eight Stereoisomers of the Germination Stimulant Strigol

Abstract: (+)-Strigol is a naturally occurring germination stimulant for the seeds of the parasitic weeds *Striga* and *Orobanche* spp. Over the past 30 years, strigol has received much attention from synthetic chemists, however, only the preparation of four out of the eight isomers have been reported so far. In this chapter, a novel synthesis of all four racemic strigol stereoisomers is presented. Subsequently, these were separated on a chiral column. All eight isomers were obtained as single compounds in high enantiopurity. The absolute configurations of the isomers were deduced from their CD-spectra. The biological activity of all eight stereoisomers was assayed with the seeds of *Striga hermonthica.*

2.1 Introduction

Parasitic weeds belonging to the genera *Striga*, *Orobanche* and *Alectra* cause severe loss of crops in Africa, Asia and the USA.[1,2] The seeds of these parasitic weeds germinate upon exposure to a chemical substance, which is present in the root exudate of a potential host plant. Strigolactones serve as semiochemicals in the rhizosphere of host plants and some non-host plants that induce the germination of seeds of root parasitic weeds. Thus far, four of these germination stimulants have been isolated viz.: (+)-strigol **1**: (+)-sorgolactone **2**, alectrol **3** and (+)-orobanchol **4** (Figure 1). The first naturally occurring germination stimulant, (+)-strigol **1,** was isolated from the root exudates of the false host cotton[3] (*Gossypium hisutum* L.) and its absolute configuration was unambiguously assigned by X-ray diffraction.[4] The synthesis of all eight stereoisomers of sorgolactone **2**[5], which has a structure closely related to strigol, has been reported by the Nijmegen

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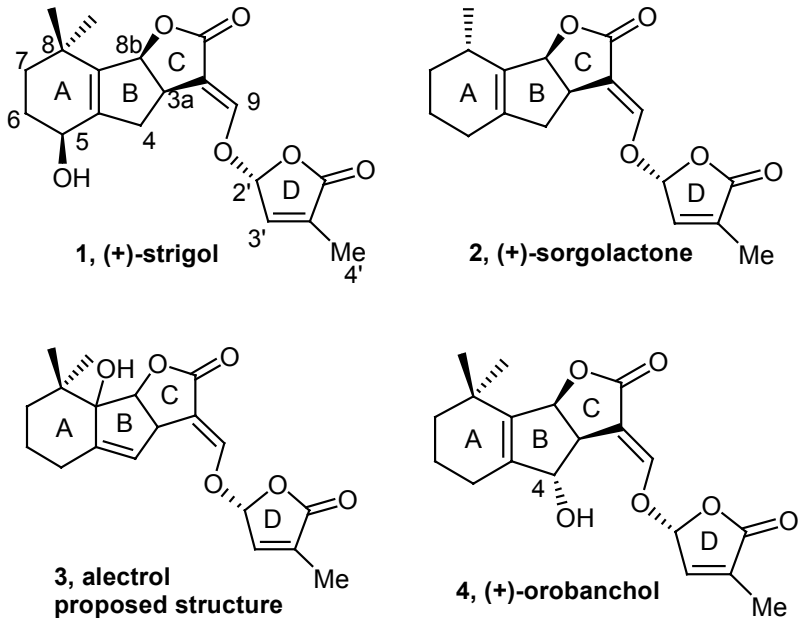
research group[6a,b] and at about the same time by the Mori group.[7] (+)-Orobanchol **3** is an isomer of strigol, which was isolated from root exudates of the host *Trifolium pratense*.[8] It was assumed by the Mori group that the secondary hydroxy group in orobanchol is attached to C-4[9] (Figure 1). Alectrol **4** was isolated for the first time from the root exudate of *Vigna unguiculata*[10] (host for *Striga* and *Alectra*). A structure has been proposed for alectrol (Figure 1), however it was recently shown by the synthesis of the proposed structure, that the spectral data do not correspond with those of the natural stimulant.[11] Strigol itself was shown to be the major *Striga* germination stimulant produced by maize (*Zea mays L.)* and proso millet (*Panicum miliaceum* L.).[12] This stimulant has been the target of extensive synthetic studies.[4,13-19,25] Thus far, the synthesis of only four of the conceivable eight stereoisomers has been accomplished in an unambiguous manner.[18,19] The synthesis of all eight stereoisomers of strigol and their germination stimulatory activity towards *Striga hermonthica* seeds, is reported in this chapter. This will provide more insight in the structure-activity relationship of these germination stimulants.

Figure 1

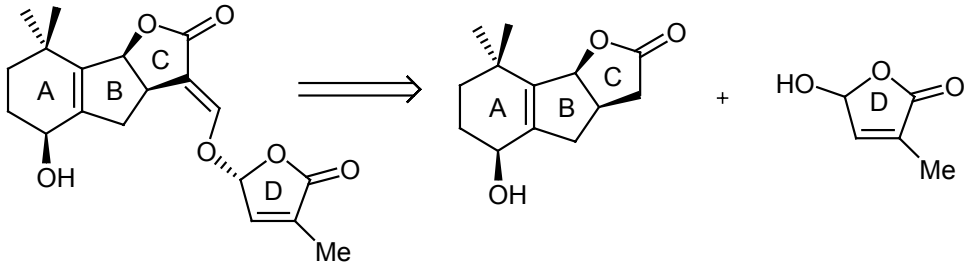


2.2 Results and Discussion

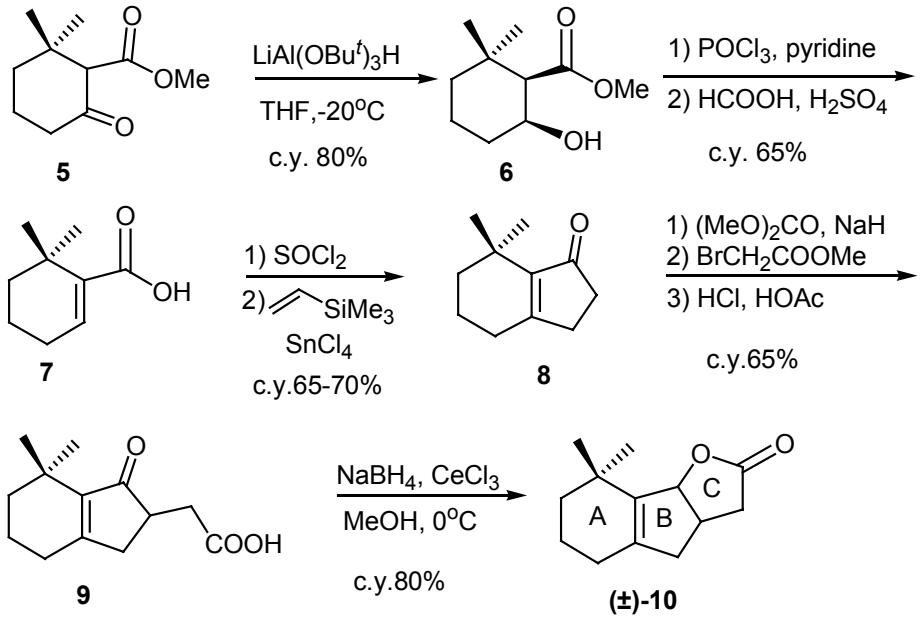
2.2.1 *Synthesis*

Retrosynthetically, there is a logical disconnection between the ABC skeleton and the D-ring (Scheme 1). Thus, the synthesis of the ABC-part was undertaken first. The starting material is compound **5** which is readily available on a multigram scale following a literature procedure.[20] Selective reduction of **5** using $LiAl(OBu^t)_3H$ gave hydroxy ester **6** as the major isomer in 80% yield. The acid **7** was obtained from **6** by dehydration and subsequent hydrolysis. The corresponding acid chloride of this acid **7** was subjected to an

Scheme 1

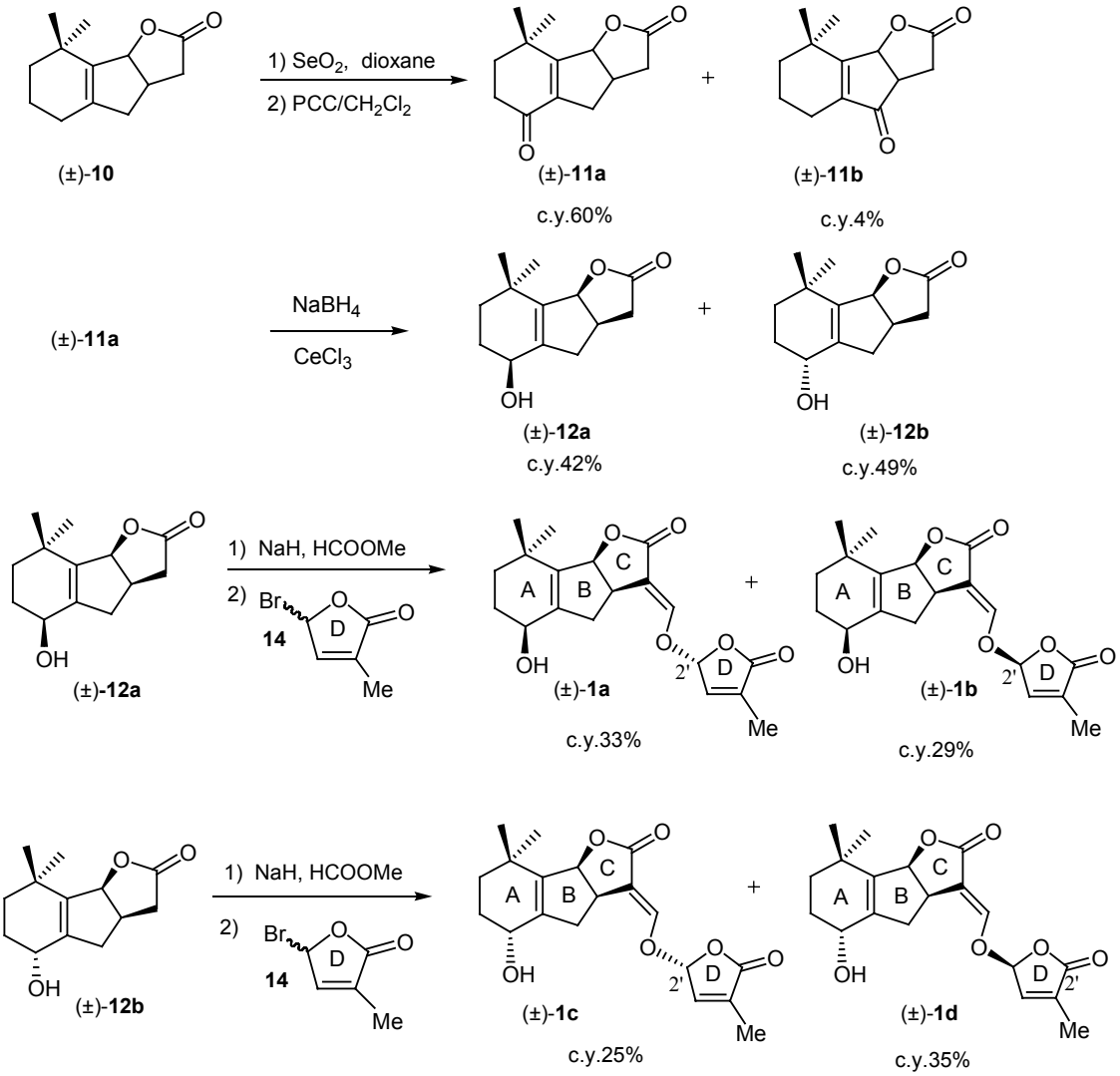


Scheme 2



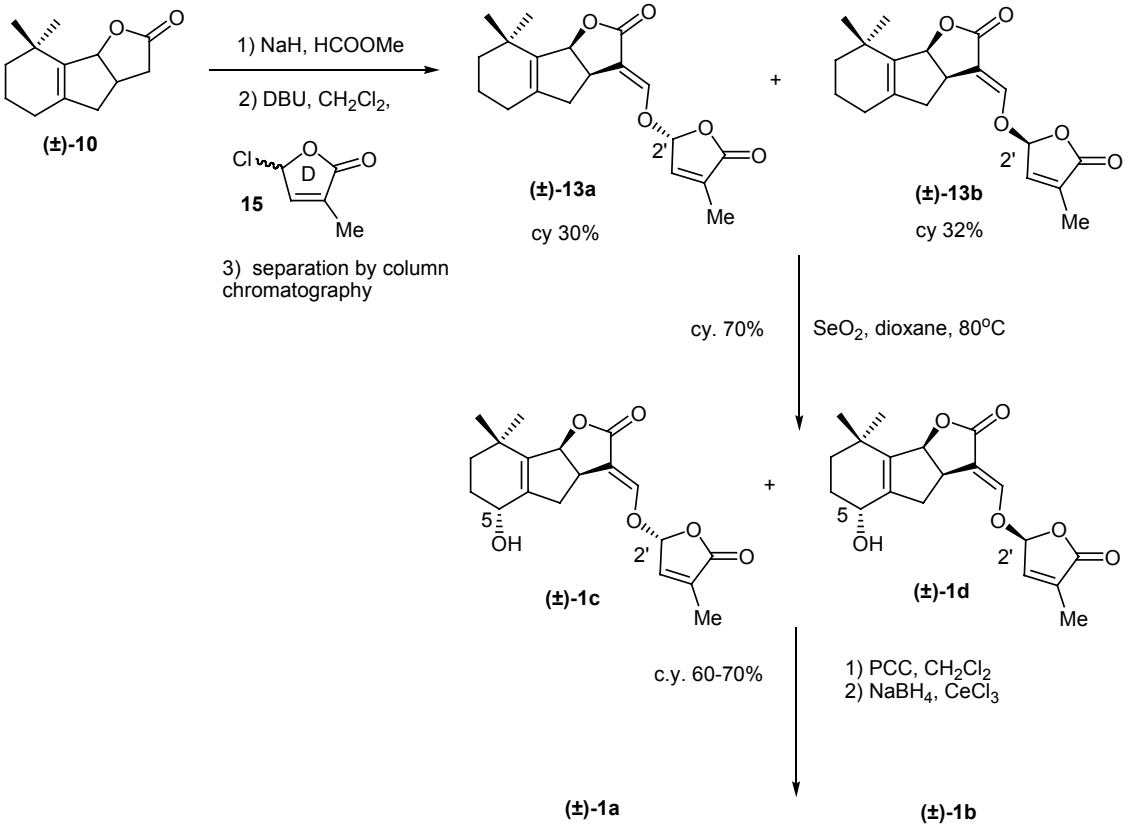
intermolecular Friedel-Crafts reaction with vinyl silane, followed by a Nazarov cyclization to give AB-fragment **8.**[21] This ketone was then converted *via* a standard procedure[22] into **9** which was subjected to Luche reduction employing sodium borohydride in the presence of cerium trichloride.[23] The resulting hydroxy acid underwent spontaneous lactonization to give the strigol ABC-fragment (±)-**10** (Scheme 2).

Scheme 3



A crucial step in the sequence to the appropriately functionalized ABC-part of strigol is the introduction of the hydroxy group in the A-ring. A highly regioselective oxidation of (±)-**10** employing selenium dioxide in dioxane, followed by oxidation with PCC furnished the isomeric ketone mixture (±)-**11a** and (±)-**11b** in a ratio of 15:1 (Scheme 3). These ketones could be separated by column chromatography. This oxidation of the intermediates alcohols to the ketones (±)-**11a** and (±)-**11b** was necessary to facilitate the chromatographic separation. Luche reduction[23] of (±)-**11a** gave **(±)-12a** and (±)-**12b** in a ratio of 1:1. After separation of the hydroxy lactones (±)-**12a** and (±)-**12b,** each isomer was formylated and coupled to the D-ring using bromo butenolide (±)-**14**.[25] Isomer (±)-**12a** gave a mixture of epimeric strigol isomers (±)-**1a** and (±)-**1b**, whereas isomer (±)-**12b** similarly produced a mixture of the racemic epimers (±)-**1c** and (±)-**1d**. In this manner, all four racemic diastereoisomers of strigol **1** had been obtained. The spectral properties of the four compounds were in full accordance with those reported previously (see Experimental Section).

Scheme 4



Alternatively, the hydroxylation of the A-ring could be accomplished after the D-ring had been coupled to the formylated ABC unit (±)-**10** (Scheme 4). The thus obtained mixture of (±)-**13a** and (±)-**13b** was separated by column chromatography. Each isomer was then oxidized with selenium dioxide to give (±)-**1c** and (±)-**1d,** respectively. Inversion of the configuration at C-5 of (±)-**1c** and (±)-**1d** to give (±)-**1a** and (±)-**1b,** respectively, could be accomplished by oxidation with PCC in dichloromethane followed by Luche reduction.[23] This stereoselectivity of the selenium dioxide oxidation of **(±)-13a** or **(±)-13b** is quite remarkable. This stereochemical outcome can be explained by assuming that in the first step, which in essence is an ene-type reaction, the approach of the double bond to SeO_2 is taking place from the opposite side of the lactone ring. Subsequent [2,3]-sigmatropic rearrangement of the selenic ester intermediate then gives the above-mentioned products.

In order to obtain enantiopure single stereoisomers several strategies have been reported previously. One involves the use of a homochiral D-ring precursor, [26,27,28,33] its coupling with the formylated racemic ABC-fragment and separation of the resulting diastereoisomers containing a latent D-ring functionality. In the final step, the D-ring had to be demasked to ultimately give the stimulant as single stereoisomers. This strategy was followed for the synthesis of all eight stereoisomers of sorgolactone.[6b] The other strategy involves synthesis of the enantiopure ABC-fragment and coupling with racemic D-ring precursor. The resulting mixture of diastereisomers was separated by chromatography to furnish two single stereoisomers of the stimulant.[13,18,24] This strategy had been used in the syntheses of (+)-strigol and its analoguos.[24] Several methods have been reported for the preparation of the ABC-part of strigol (+)-**12a** in an enantiomerically pure form. Sih and co-workers used chiral resolving agents[13] to separate (±)-**12a** into its enantiomers. Alternatively, Mori used enzymatic acetylation of (±)-**12a** with vinyl acetate in the presence of lipase AK,[19] whereas Welzel and co-wokers resolved (±)-**12a** by chromatographic separation using an HPLC chiral column of cellulose triacetate.[18] (see chapter 1). In our strategy, direct resolution of the four racemic strigol diastereoisomers (±)-**1a,** (±)-**1b,** (±)-**1c** and (±)-**1d** was accomplished by chromatographic separation using a semi-preparative cellulose carbamate Chiracel-OD® HPLC column. (+)-Strigol (**1a**), (-)-strigol (*ent*-**1a**), (+)-2'-epistrigol (**1b)**, (-)-2'-epistrigol (*ent*-**1b**) and (+)-5-epistrigol (**1c)**

were obtained directly in a high enantiomeric purity (>99% e.e.). In the case of (-)-5-epistrigol (*ent*-**1c**), (+)-2',5-bisepistrigol (**1d**) and (-)-2',5-bisepistrigol (*ent*-**1d**), the enantiopurity, as determined by analytical HPLC, was 88-90% ee. Recrystalization and repetitive chromatography afforded these stereoisomers in a high enantiopurity (Table 1). The amount of material obtained (10-30 mg) after separation was sufficient for a detailed spectral analysis and biological testing. It should be mentioned that (±)-strigol has been previously separated to its enantiomers by column of cellulose triacetate.[29] However, (+)-strigol was less pure in that case because of peak tailing of (-)-strigol and both isomers had to be rechromatographed to give the pure enantiomers. In our case, using a cellulose carbamate Chiracel-OD® HPLC column gave directly the enantiomers of strigol and 2'-epistrigol in a high enantiomerically pure form.

Table 1. Physical Data of ***1a-d*** *and their Enantiomers ent-****1a-d.***

entry	compound	configuration at C3aC5C8b	configuration at C2'[a]	ee[b] (%)	$[\alpha]_D^{23}$	HPLC[c] t_R (min)	Solvents
1	**1a**	RSS	R	>99	+ 260	16.4	hexane/2-propanol
2	*ent*-**1a**	SRR	S	>99	-261	7.1	(1/1)
3	**1b**	RSS	S	>99	+144	11.5	hexane/2-propanol
4	*ent*-**1b**	SRR	R	>99	-142	9.4	(1/1)
5	**1c**	RSS	S	>99	+311	18.8	hexane/2-propanol
6	*ent*-**1c**	SRR	R	>99	-308	20.3	(7/3)
7	**1d**	RSS	R	>99	-166	18.5[d]	hexane/$CHCl_3$
8	*ent*-**1d**	SRR	S	>99	+168	16.1	(6/4)

a. Configuration at C'2 was deduced from CD-spectra. b. Enantiomeric excesses were determined by chiral HPLC. c. Semi-preparative cellulose carbamate Chiracel-OD® HPLC column. Flow rate: 3 mL/min. d. Flow rate: 6 mL/min.

Considerable difference in retention time was observed between the two enantiomers of strigol and epistrigol (Table 1, entries 1,2). (-)-Strigol eluted after 7.1 minutes, whereas (+)-strigol eluted after 16.4 minutes. (-)-2'-epistrigol eluted after 11.5 minutes and (+)-2'-epistrigol after 9.4 minutes (Table 1, entries 3,4). Only three of the eight stereoisomers, viz. (-)-5-epistrigol (*ent*-**1c**), (+)-2',5-bisepistrigol (**1d**) and (-)-2',5-bisepistrigol (*ent*-**1d**), had to be chromatographed repetitively on a HPLC column because of the presence of the other enantiomer as an impurity which resulted in peak

tailing. Cellulose carbamate Chiracel-OD® HPLC column showed a broad selectivity in the resolution of strigol analogous like deshydroxy strigol[30], methyl GR-24[31] and sorgolactone.[6b] This method, involving the separation of enantiomers on a chiral preparative HPLC column is convenient when small amounts of material are required for biological testing. In addition, only minimal amount of materials were lost during this separation, whereas in the previously reported methods two additional steps, protection and deprotection are required for the resolution which reduces the yield of the end product. Finally, the absolute configurations of **1a**, (-)-*ent*-**1a**, **1b,** and *ent*-**1b** were determined by comparing of their physical (mp and $[\alpha]_D$) and spectral (^{1}H NMR, ^{13}C NMR and CD) properties with those reported earlier (see Experimental Section). The data were in full accordance with those reported previously. In the case of **1c**, *ent*-**1c, 1d** and *ent*-**1d,** deduction of the absolute stereochemistry at C2' was not possible using NMR techniques. Therefore, circular dichroism spectrometry was used to determine the absolute configuration at C2' of **1c**, *ent*-**1c, 1d** and *ent*-**1d**. Figure 2-5 shows the CD-spectra of all eight stereoisomers of strigol.

*Figure 2-5 CD-spectra of strigol stereoisomers **1a-d** and their enantiomers ent-**1a-d** using acetonitrile as the solvent.*

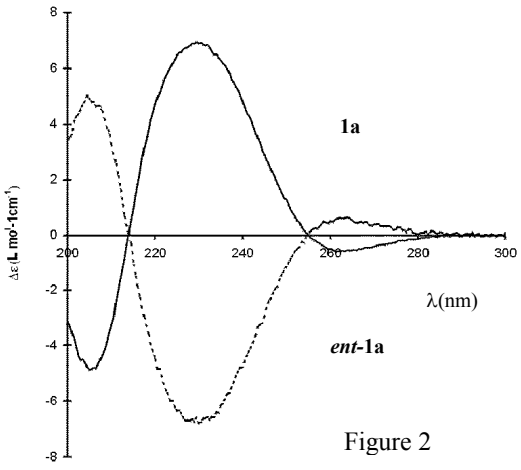


Figure 2

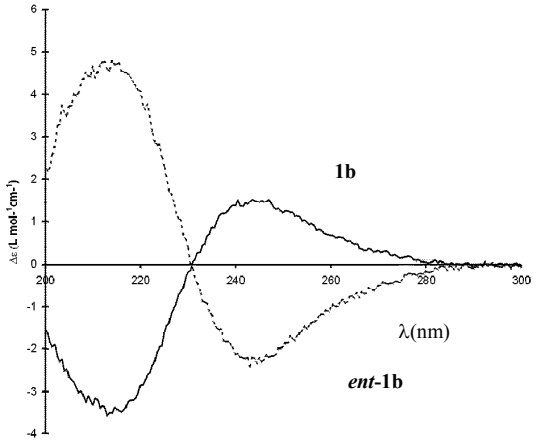


Figure 3

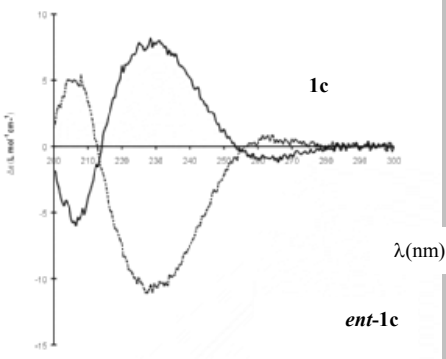


Figure 4

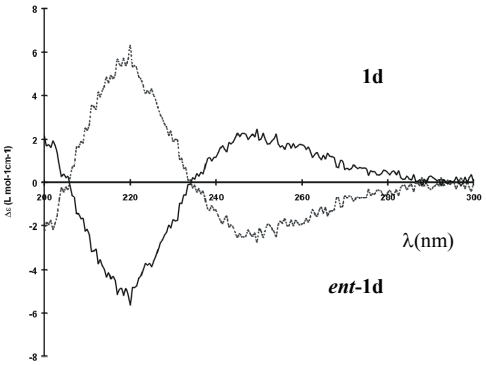


Figure 5

Welzel and co-workers[32,33] demonstrated that the sign of the cotton effect around 270 nm could be directly correlated with the stereochemistry at C2', í.e. a negative sign at this wavelength corresponds with the C2'(*R*) configuration, whereas a positive sign corresponds with the C'2(*S*) configuration. This rule has proven its value in several series of strigolactones, in particular for GR 28,[34] desmethylsorgolactone[35] and sorgolactone.[6b] In this manner, the absolute configuration of all eight stereoisomers of strigol was assigned unambiguously.

Biological Activity

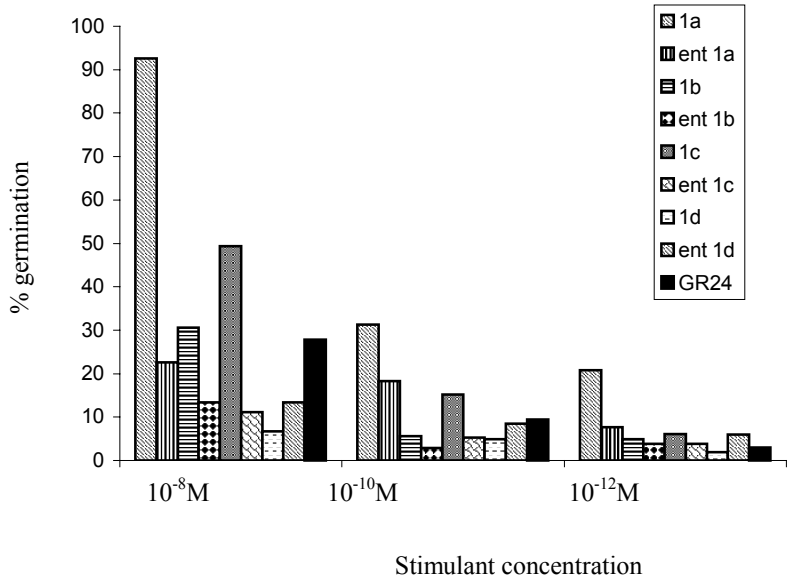
The germination stimulatory activity of all eight stereoisomers of strigol **1a-d** and *ent.* **1a-d**, was assayed using seeds of *Striga hermonthica*.

Table 2. Germination percentages for seeds of *S. hermonthica* after exposure to solutions of $1x10^{-8}$, $1x10^{-10}$,$1x10^{-12}$ mol/L of strigol diastereoisomers **1a-d** and *ent*-**1a-d** and the control GR24

entry	compound	configuration at C3aC5C8b	at C2'	% germination [a] ±SE at a concentration of		
				10^{-8} mol/L	10^{-10} mol/L	10^{-12}mol/L
1	**1a**	*RSS*	*R*	92.6 ± 5.6	31.3 ± 3.3	18.7 ± 3.4
2	*ent*-**1a**	*SRR*	*S*	22.5 ± 3.8	18.2 ± 1.42	5 ± 3.8
3	**1b**	*RSS*	*S*	30.6 ± 6.7	5.6 ± 0.6	5.0 ± 0.4
4	*ent*-**1b**	*SRR*	*R*	21.4 ± 7.7	2.9 ± 0.9	3.8 ± 0.9
5	**1c**	*RRS*	*R*	49.3 ± 7.4	15.1 ± 1.0	6.1 ± 0.9
6	*ent*-**1c**	*SSR*	*S*	11.1± 0.6	5.2 ± 1.2	3.9 ± 1.3
7	**1d**	*RRS*	*S*	6.7 ± 1.0	4.9 ± 0.2	1.9 ±1.5[c]
8	*ent*-**1d**	*SSR*	*R*	14.4 ± 2.8	9 .8 ± 7.5	8.5 ± 0.8
9	GR24[b]	racemic		27.8 ± 7.0	9.4 ± 6.3	3.0 ± 0.3 [c]

[a] Data presented are the mean ± SE of one representative experiment. [b] Equimolar mixture of two racemic diastereoisomers. [c] Not significantly different from aqueous control (without stimulant).

*Figure 6. Bar representation of the percentage of germinated seeds of S. hermonthica after exposure to various concentrations of strigol stereoisomers **1** and the control GR24.*



The data presented in table 2 and figure 6 show a significant difference in germination stimulatory activity between the eight stereoisomers of strigol. At all three concentrations 10^{-8},10^{-10} and 10^{-12} M, (+)-strigol **1a**, having the "natural" absolute stereochemistry, is by far the most active one.

At a concentration of 10^{-8}M, **1a** induced germination for more than 90% of S. *hermonthica* seeds, whereas *ent*-**1a** induced only 20% germination of these seeds. Furthermore, the "natural" isomer **1a,** was still active at a concentration as low as 10^{-12}M, whereas the activity of all other isomers was much lower. Isomer **1c** exhibits a relatively high activity compared to the other isomers (table 2, entry 6). This isomer possesses the same absolute configuration at the CD-rings as the "natural" strigol (**1a**). This observation is in line with the results reported by Thuring *et. al*. regarding the importance of the absolute stereochemistry at the CD-part for bioactivity.[6b,35,38] Müller *et al.*[10] determined the stimulatory activity of (+)-strigol on seeds of *S. hermonthica* and reported for similar concentrations 90% ,60%, and no activity, respectively. The data reported here indicate that the positive control GR24 exhibits low percentage of germination compared to the previous reports.[6b] The data that were reported earlier by the Nijmegen research group[6b] were 60% and 38% for the concentrations of 10^{-8}M and 10^{-10}M, respectively. In the present case these bioassays only show 27.8 % and 21% percentage germination at similar concentrations. The low germination activity can be explained by the fact that these bioassays were performed in October in which period germination of the seeds is relatively low compared to the summer months June-August. It has indeed been demonstrated by Magnus *et al*.[37] that the germination response of parasitic seeds is seasonally dependent. However, although the overall response is rather low, the same trends are observed as for the other series of GR24[38] and sorgolactone[6b] stereoisomers, namely the natural stereoisomer being the best, and its mirror image being the worst stimulant.

2.3 Conclusions

The aim of this work was to synthesize all eight stereoisomers of strigol in enantiopure form. By a novel synthesis, the four racemic strigol stereoisomers were synthesized and the racemates were subsequently separated on a chiral column. This is the first time that

all eight stereoisomers of strigol have been obtained as single compounds. The absolute configurations of **1a**, (-)-*ent*-**1a**, **1b,** and *ent*-**1b** were determined by comparing of their physical (mp and $[\alpha]_D$) and spectral (^{1}H NMR, ^{13}C NMR and CD) properties with those reported earlier. The data were in full accordance with those reported previously. In the case of **1c**, *ent*-**1c, 1d** and *ent*-**1d,** the absolute stereochemistry at C2' was deduced on the basis of circular dichroism spectrometry.

The bioactivities of all eight stereoisomers of strigol were determined using the seeds of *S. hermonthica*. A significant difference in germination stimulatory activity between the stereoisomers was observed. Strigol with the natural absolute configuration was the most active, whereas, its enantiomer was much less active at a concentration of 10^{-8} mol/L.

The isomer **1c** having the same absolute stereochemistry at the CD-junction, that is at the C-2' carbon atom, as "natural" strigol (**1a**) exhibits higher germination stimulatory activity compared to the other strigol stereoisomers. This observation is in line with the bioactivity for the stereoisomers of sorgolactone,[6] desmethylsorgolactone[35] and GR24.[38]

2.4 Experimental

2.4.1 Synthesis

General. ^{1}H NMR and ^{13}C NMR (300 MHz) spectra were recorded on a Bruker AC 300 spectrometer, using TMS as internal standard. Mps were measured with a Reichert thermopan microscope and are uncorrected. IR spectra were recorded on a Bio-Rad FTS-25 instrument. For mass spectra a double focusing VG7070E mass spectrometer was used. GC-MS spectra were run on a Varian Saturn 2 GC-MS ion-trap system. Separations were carried out on a fused-silica capillary column (DB-5, 30 m x 0.25 mm), helium was used as the carrier gas and electron impact (EI) was used as ionization mode. Elemental analyses were conducted on a Carlo Erba Instrument CHNSO EA 1108 element analyzer. For the determination of optical rotations a Perkin-Elmer 241 polarimeter was used. Thin layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F254 plates (0.25 mm). Column chromatography was performed with Merck Kieselgel 60. Preparative HPLC separation of racemates **1a**, **1b**, **1c** and **1d** was carried out on a semi-preparative Chiralcel OD (10 μm) cellulose carbamate column (Daicel Chemical Industries Ltd., 1 x 25 cm) using mixtures of hexane/2-propanol 1/1 for (±)-**1a** and (±)-

1b, hexane/2-propanol 7/3 for (±)-**1c** and hexane/chloroform 6/4 for (±)-**1d**. Products were detected with a Merck-Hitachi L-4000 UV detector at 240 nm. Enantiomeric excesses and purities of the stereoisomers **1a-d** and *ent*-**1a-d** were determined by analytical HPLC using a Cyclobond I 2000 RSP column and CH_3CN/ 0.1% TEAA pH 4.1 (1/3) as the eluent. CD-spectra were recorded using a Jasco spectrophotometer.
All solvents were dried using standard methods. Reagents were obtained from commercial suppliers and were used without further purification.

Methyl (1*S, 6*R**)-6-hydroxy-2,2-dimethylcyclohexane-1-carboxylate (6)**

To a stirred suspension of lithium tri-*tert*-butoxyaluminum hydride (50 g, 0.197 mol) in dry THF (120 mL) cooled to –20°C, **5**[20](20 g, 0.1086 mol) in dry THF (20 mL) was added and the resulting mixture was stirred under nitrogen at –20 ^{0}C. After 2 h of stirring at the same temperature the solvent was removed in vacuo and the residue was dissolved in a mixture of HCl (6N) and ethyl acetate. The aqueous layer was extracted with ethyl acetate (3x30 mL) and the combined organic layers were washed with saturated $NaHCO_3$ and brine, then dried ($MgSO_4$) and concentrated in vacuo to give crude hydroxy ester **6** (19.2 g, 80%) as a major isomer as yellow oil, which was sufficiently pure for further reactions.

6,6-Dimethyl-1-cyclohexene-1-carboxylic acid (7)

This compound was prepared from the corresponding ester following the procedure described by Sugimoto *et al*[6b]. The ester was obtained as a colorless oil (29.0 g, 75-80%) and was sufficiently pure for further reactions. To the solution of the ene ester (29.0 g, 0.188 mol) in formic acid (300 mL) a catalytic amount of concentrated sulfuric acid was added and the resulting solution was refluxed for 12 h. After cooling, 20 mL of H_2O was added and the aqueous layer was extracted with ethyl acetate (2x40 mL). The combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated in vacuo to give white-yellow crystals of the acid **7**. Recrystallization from hexane/diisopropyl ether gave colourless crystals (65% yield from **6**) mp 158-160°C.
^{1}H NMR($CDCl_3$, 100 MHz)): δ = 1.23 (s, 6H, 2xMe), 1.46–1.67 (m, 4H, CH_2), 2.10-2.25 (m, 2H, $\underline{CH_2}$-CH=C), 7.01-7.25 (t, 1H, $\underline{CH}$=C), 11.95 (bs, 1H).

Anal. calcd for $C_9H_{14}O_2$ (154.209) : C, 70.10; H, 9.15. Found: C, 70.24; H, 9.23.

7,7-Dimethyl-2,3,4,5,6,7-hexahydro-1*H*-1-indenone (8)

This compound was prepared from the acid **7** analogous to the procedure described by Sugimoto *et al.*[6b] Yield: 65-70%. An analytically pure sample was obtained by recrystallization from diisopropyl ether. mp 47-49^{o}C (Lit.[25] mp 45^{o}C)

^{1}H and ^{13}C NMR ($CDCl_3$, 100 MHz): these spectral data were in full accord with those reported in ref.[25]

Anal. calcd for $C_{11}H_{16}O$ (164.2) : C, 80.44; H 9.82. Found :C, 80.30; H, 9.84.

8,8-Dimethyl-3, 3a,4,5,6,7,8,8b-octahydrohydro-2*H*-1-indeno [1,2-*b*]furan-2-one (±)**10**. This compound was prepared from **8** analogous to the procedure described by Sugimoto *et al*.[6] Yield: 52% from **8**. Crude oil (95% pure by GC). This compound was sufficiently pure for further reaction. ^{1}H and ^{13}C NMR ($CDCl_3$, 300 MHz) spectra were in full accord with those reported previously.[16]

8,8-Dimethyl-3,3a,4,5,6,7,8,8d-octahydro-2*H*-indeno[1,2-*b*]furan-2,5-dione (±)-11a

A solution of the lactone (±)-**10** (0.7 g, 3.4 mmol) and SeO_2 (0.45 g, 4.05 mmol) in dioxane (50 mL) was heated for 4 h at 80^{0}C. After cooling, the reaction mixture was filtered through Hyflo Super-Cell® and the filter cake washed several times with ether (20 mL). Solvents were removed in vacuo and the crude orange oil (0.669 g) was used as such without purification for further reactions. To a stirred solution of this crude mixture in CH_2Cl_2 (20 mL) pyridinium cholorochromate (PCC) was added in portion until no starting material was detected any more by TLC. Filtration and concentration in vacuo gave a crude mixture which was subjected to column chromatography (silica gel; hexane/EtOAc; 2/1) to give the fast isomer (±)-**11a** (0.435 g, 60% as colorless crystals; mp 86-87 (hexane/diethyl ether) [Lit.[19] 85-86^{0}C] and the slow isomer (±)-**11b** (0.026 g, 4%) as colorless crystals**;** mp 70-71^{0}C (hexane/diethyl ether) [Lit.[9,19] 69-71^{0}C]

^{1}H and ^{13}C NMR ($CDCl_3$, 300 MHz): these spectral data were in full accord with those reported in ref.[9,15,19]

5-Hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydro-2*H*-indeno[1,2-*b*]furan-2-one (±)-12a

Brooks' procedure was used.[15] To a stirred solution of α,β-unsaturated lactone (±)-**11a** (0.24 g, 1.09 mmol) in ethanol (25 mL), $CeCl_3.7H_2O$ (0.46 g, 1.09 mmol) was added, followed by slow addition of $NaBH_4$ (0.0412 g, 1.09 mmol) at 0^0C. After stirring for 30 min, the reaction mixture was quenched by dropwise addition of 1N HCl and then extracted with CH_2Cl_2 (2 X 25 mL). The organic layer was washed with brine and dried ($MgSO_4$). Evaporation of the solvent gave a crude product. (±)-**12a** which recrystallized selectively from ether solution of the mixture to give colorless needles (0.10 g, 42%); mp 146^0C Lit.[15] 142-144^0C and (±)-**12b** (0.12 g, 49%) as an oil.

^{1}H and ^{13}C NMR ($CDCl_3$, 300 MHz): these spectral data were in full accord with those reported in ref.[13-17,25]

(±)-1a and **(±)-1b**

According to Brooks' procedure[15], starting from hydroxylactone **(±)-12a** (100 mg, 0.44 mmol) **(±)-1a** obtained as a white solid. Yield: 52 mg (33%); mp 205-206^{o}C (hexane/EtOAc) (ref.[25] mp 202-205^{o}C) and **(±)-1b** Yield: 45 mg (29 %); mp 177-178^0C (Lit.[25] mp 178-180^0C).

^{1}H NMR, ^{13}C NMR, MS, and IR spectra where in full accord with the literature data.[15, 25]

(±)-1c and **(±)-1d**

These compounds were prepared from **(±)-12b** following the procedure used for the preparation of **(±)-1a** and **(±)-1b**.

Compound **(±)-1c;** Yield 25%; mp 157-158^0C (colorless crystals from hexane/EtOAc) (Lit.[25] mp 156-158^0C, Lit.[16] mp 150-151^0C);

(±)-1d; Yield 35%; mp 186-188 ^{o}C (colorless crystals from hexane/EtOAc) (Lit.[16,25] mp 188-190^{o}C).

^{1}H NMR, ^{13}C NMR, MS, and IR spectra where in full accord with those reported in ref.[16,25]

Determination of the enantiomeric purity of 1a, *ent*-**1a, 1b** and *ent*-**1b:**
HPLC [column: Chiralcel OD, 4.6 mm x 25 cm; solvent: hexane/iPrOH (1/1), flow rate: 0.5 mL/min; detector: 240 nm; temperature; r.t.], t_R = 10.7 min [>99, **1a**], t_R = 22.5 min [>99, *ent* **1a**], t_R = 9.2 min [>99, **1b**], t_R = 11.0 min [>99, *ent* **1b**],

(+)-(3a*R*,5*S*,8b*S*,2'*R*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo-2'furanyloxymethylene] -5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (1a) [(+)-Strigol]
22 mg; mp 191-193°C (colorless crystals from pentane/CH_2Cl_2) (Lit.[18] mp 191-193°C) $[\alpha]_D^{23}$= +260 (c = 0.25, $CHCl_3$) [Lit.[7c] $[\alpha]_D^{25}$ = +271 (c = 0.5, $CHCl_3$)], [Lit.[13] $[\alpha]_D^{25}$ = +293.0 (c = 0.15, $CHCl_3$)], [Lit.[15] $[\alpha]_D$ = +270 (c = 0.2, $CHCl_3$)], $[\alpha]_D^{20}$= +262.7 (c = 0.69, $CHCl_3$)]
^{1}H NMR($CDCl_3$, 400 MHz): δ = 1.10 (s, 3H, 8-Me), 1.17 (s, 3H, 8-Me), 1.42–1.49 (ddd, 1H, *J* = 13.5, *J*' = 11.3, *J*'' = 2.8 Hz, 7-H), 1.52-1.60 (m, 1H', 7-H'), 1.64-1.73 (m, 1H, 6-H), 1.95-2.01 (ddd, 1H, *J* = 13.4, *J*' = 7.1, *J*'' = 3.1 Hz, 6-H'), 2.00-2.03 (t, 3H, *J* =1.5 Hz, 4'-Me), 2.70-2.73 (m, 2 H, 4-CH_2,), 3.61-3.67 (dtd,1 H, *J* = 11.9, *J*' = 4.0, *J*'' = 2.5 Hz, 3a-H), 4.10-4.15 (t, 1 H, *J* =6.1, 5-H), 5.50-5.53 (d, 1H, *J* = 7.5 Hz, 8b-H), 6.14-6.15 (m, 1 H, 2'-H), 6.92-6.94 (m, 1 H, 3'-H), 7.45-7.46 (d, 1H, *J* =2.4 Hz, 9-H)
^{13}C NMR($CDCl_3$, 400 MHz): δ = 10.7, 27.5, 27.6, 29.7, 32.4, 36.6, 37.0, 37.8, 67.4, 87.9, 100.6, 113.7, 135.9, 140.9, 142.5, 142.6, 150.6, 170.2, 171.4
These spectral data were in full accord with those reported in ref. [18,19]
HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1414.

(-)-(3a*S*,5*R*,8b*R*,2'*S*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo-2'furanyloxymethylene]- 5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (*ent*-1a) [(-)-Strigol]
14 mg; mp 192-193°C (colorless crystals from pentane/CH_2Cl_2) [(Lit.[19] mp 194-196°C), [ref.[13] mp 193-194°C] $[\alpha]_D^{23}$= -261 (c = 0.25, $CHCl_3$) [ref.[19] $[\alpha]_D^{25}$ = -277 (c = 0.37, $CHCl_3$)], [ref.[13] $[\alpha]_D^{25}$ = -279 (c = 0.11, $CHCl_3$),
^{1}H and ^{13}C NMR ($CDCl_3$, 400 MHz) spectra were identical with those of (+)-**1a**
HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1409.

(+)-(3a*R*,5*S*,8b*S*,2'*S*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo 2'furanyloxymethylene] -5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (1b) [(+)-2'-Epistrigol]

30 mg; mp 159-160^{0}C (colorless crystals from benzene/pentane) [Lit.[19] mp 158-159^{0}C]. $[\alpha]_D^{23}$ +144 (c =0.3 ,$CHCl_3$) [Lit.[19] $[\alpha]_D^{24}$ +145 (c = 1.24 ,$CHCl_3$)]

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.08(s, 3H, 8-Me), 1.14(s, 3H, 8-Me), 1.39–1.47 (ddd, 1H, *J* = 13.5, *J*' = 11.0, *J*'' = 3.5 Hz, 7-H), 1.52-1.60 (m, 1H', 7-H'), 1.63-1.72 (m, 1H, 6-H), 1.78-1.86 (bs, 1H, OH), 1.92-2.2 (m, 1H, 6-H', and t, 3 H, *J* =1.5 Hz, 4'-Me), 2.62-2.75 (m, 2 H, 4-CH_2), 3.58-3.66 (m,1 H, 3a-H), 4.04-4.10 (t, 1 H, J = 6.5 Hz, 5-H), 5.46-5.52 (d, 1H, J = 8.0 Hz, 8b-H), 6.14-6.15 (m, 1 H, 2'-H), 6.90-6.93 (m, 1 H, 3'-H), 7.40-7.44 (d, 1H, *J* =2.5 Hz, 9-H)

^{13}C NMR ($CDCl_3$, 400 MHz): δ = 10.7, 27.5, 27.6, 29.6, 32.3, 36.5, 37.0, 37.9, 67.2, 88.0, 100.4, 113.8, 135.8, 141.1, 142.2, 142.8, 150.3, 170.2, 171.5

These spectral data were in full accord with those reported in ref. [19]

HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1420.

(-)-(3a*S*,5*R*,8b*R*,2'*R*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo-2'furanyloxymethylene] -5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (*ent*-1b) [(-)-2'-Epistrigol]

18 mg; mp 160-161^{0}C (colorless crystals from CH_2Cl_2/pentane [Lit. [19] mp 146-148^{0}C (CH_2Cl_2/pentane), 155-156^{0}C (benzene/pentane), $[\alpha]_D^{23}$ -142 (c = 0.25 ,$CHCl_3$) [Lit. [19] $[\alpha]_D^{25}$ -141 (c = 1.38, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 400 MHz) spectra were identical with those of (+)-**1b.**

HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1410.

Determination of the enantiomeric purity of 1c, *ent*-1c, 1d and *ent*-1d: HPLC [column: cyclobond I 2000 RSP, 4.6 mm x 25 cm; solvent: CH_3CN/0.1%TEAA, pH 4.1 (1/3), flow rate: 0.5 mL/min; detector: 240 nm; temperature; r.t.] ; t_R = 15.1 min [>99%, **1c**], t_R = 17.63 min [99%, *ent* **1c**], t_R = 17.7 min [>99%, **1d**], t_R = 14.4 min [99%, *ent* **1d**].

(+)-(3a*R*,5*R*,8b*S*,2'*S*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo-2'furanyloxymethylene] -5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (1c) [(+)-5- Epistrigol]

10 mg; mp 141-142°C (colourless crystals from pentane/ether), $[\alpha]_D^{23}$ +311 (c = 0.128 ,$CHCl_3$)

^{1}HNMR ($CDCl_3$, 400 MHz): δ = 1.11 (s, 3H, 8-Me), 1.14 (s, 3H, 8-Me), 1.42–1.48 (ddd, 1H, *J* = 13.5, *J*' = 8

, *J*'' = 3.3 Hz, 7-H), 1.51-1.59 (m, 2H', 7-H', OH), 1.66-1.73 (m, 1H, 6-H), 1.94-2.00, (m, 1H, 6-H'), 2.00 (t, 3H, *J* = 1.5 Hz, 4'-Me), 2.35-2.40 (d, 1 H, *J*' = 16.0 Hz, 4-CH_2), 3.05-3.12 (dd,1 H, *J*' = 17.2, *J*'' =9.2, 4'-CH_2), 3.61-3.66 (m, 1H, 3a-H) 4.14-4.17 (t, 1 H, *J* = 4.8 Hz, 5-H), 5.52-5.54 (dd, 1H, *J'* = 7.6, *J''* = 2.0, Hz, 8b-H), 6.14-6.15 (m, 1 H, 2'-H), 6.91-6.93 (m, 1 H, *J* = 1.5 Hz, 3'-H), 7.43-7.44 (d, 1H, *J* =2.4 Hz, 9-H)

^{13}C NMR($CDCl_3$, 400 MHz): δ = 10.7, 27.3, 27.4, 29.4, 32.4, 35.2, 36.6, 38.3, 66.0, 88.1, 100.4, 113.9, 136.1, 140.9, 141.9, 143.05, 150.0, 170.15, 171.4

HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1403.

(-)-(3a*S*,5*S*,8b*R*,2'*R*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo-2'-furanyloxymethylene] -5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (*ent*-1c) [(-)-5- Epistrigol]

12 mg; mp 142-143 °C (colorless crystals from pentane/ether),

$[\alpha]^{23}_D$ -308 (c = 0.09 ,$CHCl_3$)

^{1}H and ^{13}C NMR ($CDCl_3$, 400 MHz) spectra were identical with those of (+)-**1c**

HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1408.

(+)-(3a*R*,5*R*,8b*S*,2'*R*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo,2'furanyloxymethylene] -5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (1d) [(+)-2,5- Bisepistrigol]

14 mg; mp 95-96°C (colorless crystals from pentane/ether),

$[\alpha]^{23}_D$ -166 (c = 0.12 ,$CHCl_3$).

^{1}H NMR data ($CDCl_3$, 400 MHz) were superimposable with those of **1c.**

^{13}C NMR($CDCl_3$, 400 MHz): δ = 10.7, 27.3, 27.4, 29.3, 32.4, 35.2, 36.5, 38.3, 65.9, 88.1, 100.5, 113.9, 135.9, 140.9, 141.9, 142.59, 150.4, 170.2, 171.4
HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1412.

(-)-(3a*S*,5*S*,8b*R*,2'*S*)-3-[(E)-2',5'-Dihydro-4'-methyl-5'-oxo-2'furanyloxymethylene] -5-hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydroindeno[1,2-*b*]furan-2-one (*ent*-1d) [(-)-2,5- Bisepistrigol]

10 mg; mp 93-94^{o}C (colorless crystals from pentane/ether), $[\alpha]^{23}_D$ +168 (c = 0.07 ,$CHCl_3$).
^{1}H and ^{13}C NMR ($CDCl_3$, 400 MHz) spectra were identical with those of (+)-**1d.**
HRMS: calcd for $C_{19}H_{22}O_6$ 346.1416. Found: 346.1412.

(±)-13a and **(±)-13b**

Compound **(±)-10** was formylated with ethyl formate and NaH as described by Brooks et al.[15] A solution of the formylated product (80 mg, 0.34 mmol) in CH_2Cl_2 (15 mL), DBU (0.38 mL, 0.37 mmol), chlorobutenolide (54 mg, 41 mmol) in CH_2Cl_2 (5 mL) was added dropwise at r.t. The reaction stirred for 2 h and then solvent was evaporated in vacuo to give a crude mixture which was readily separated by column chromatography (silica gel; hexane/EtOAc; 3/1) to give (±)-**13a** (34 mg, 30%) as colorless crystals; mp 139-140^{0}C [ref.[16] mp 139^{0}C] and (±)-**13b** as colorless crystals (36 mg, 32%) mp 146-147^{0}C.

An alternative method for **(±)-1a, (±)-1b**, **(±)-1c** and **(±)-1d.** A solution of (±)-**13a** (50 mg, 0.15 mmol) and SeO_2 (50 mg, 0.45 mmol) in dioxane (15 mL) was heated for 5 h at 80^{0}C. After cooling, the reaction mixture was filtered through Hyflo Super-Cell® and the filter cake washed several times with ether (20 mL). Solvents were removed in vacuo and the crude orange oil subjected to column chromatography (silica gel; hexane/EtOAc; 3/1) afforded (±)-**1c** as colorless crystals (0.37 mg, 70%). Its physical and spectral data were identical to those obtained previously.

Compound (±)-**1a** was obtained from (±)-**1c** by oxidation using PCC in CH_2Cl_2 in the same manner is described for the reduction of (±)-**10**. The crude mixture α,β-unsaturated

ketone was sufficiently pure for further reactions. This ketone was reduced to the alcohol Compound (±)-**1a** in the same manner as described for reduction of (±)-**11a** to (±)-**12a** using Luche reduction.[24] Yield 60-70%.

Compound (±)-**1b** was obtained from (±)-**13b** in the same manner as **(**±)-**1a** from (±)-**13b**. Its physical and spectral data were identical to those reported previously.

2.4.2 Biological Activity[36]

Plant material. Seeds of *Striga hermonthica* (Del.) Benth. were collected from Sorghum (Sorghum bicolor (L.) Moench) on the Gezira Research station, Sudan in 1994. The seeds were stored in glass vials in the dark at room temperature until used in germination tests.

Preparation of test solutions. A compound to be tested was weighed out very accurately to the amount of 1.0 mg, dissolved in 5 mL of acetone p.a. and diluted with demineralized water to 50 mL. These stock solutions of approximately 10^{-4} mol L^{-1} (the exact concentration depending on the molecular mass of the compound used) were further diluted with demineralized water to obtain test solutions with concentrations of 2×10^{-8} ,2×10^{-10} and 2×10^{-12} mol L^{-1} . All solutions were prepared just before use.

Bioassays.[36] All bioassays were performed at the Department of Organic Chemistry University of Nijmegen. For surface sterilization all seeds were exposed for 5 min to 50% (v/v) aqueous solutions of commercial bleach (2% hypochlorite). Subsequently, the seeds were thoroughly rinsed with demineralized water and air-dried. For conditioning the seeds were spread on glass fiber filter paper disks (8 mm diameter, approximately 60-100 seeds per disk) in Petri dishes, wetted with demineralized water and stored in the dark at 30 °C. Thereafter the conditioning water was removed and conditioned seeds were placed in new Petri dishes and exposed to test solution. After incubation for 24 hours in the dark at the indicated temperatures the percentages of germination seeds were determined under a microscope. Seeds were considered to be germinated if the radicle protruded through the seed coat. In each test series an aqueous solution of 0.1% acetone was included as a negative control. For full details of the bioassays, see Mangnus *et al.*[36,37]

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CHAPTER 3

Synthesis of the Germination Stimulants (±)-Orobanchol and (±)-Strigol via an Allylic Rearrangement

Abstract: (+)-Strigol and (+)-orobanchol are naturally occurring germination stimulants for the seeds of parasitic weeds *Striga* and *Orobanche* spp. The synthesis of strigol **1**, the first natural germination stimulant ever isolated, has been a challenge for many synthetic chemists. Nevertheless, there is still room for more efficient syntheses. The synthesis of (+)-orobanchol **3** was recently reported by Mori et al. The structure elucidation was mainly based on GC-MS data, since only a minute amount of stimulant could be obtained from this natural source. This chapter describes an efficient synthesis of **(±)-**orobanchol and **(±)-**strigol via allylic rearrangement of lactones **(±)-12** and **(±)-14,** respectively.

3.1 Introduction

Strigolactones are important semiochemicals in the life cycle of the devastating root parasites *Striga* spp. and *Orobanche* spp.[1] These compounds serve as germination agents of seeds of these parasitic weeds. After germination, attachment to the roots of suitable host plants take place resulting in the development of the parasitic weed at the expense of the nutrients of the hosts.[2,3]

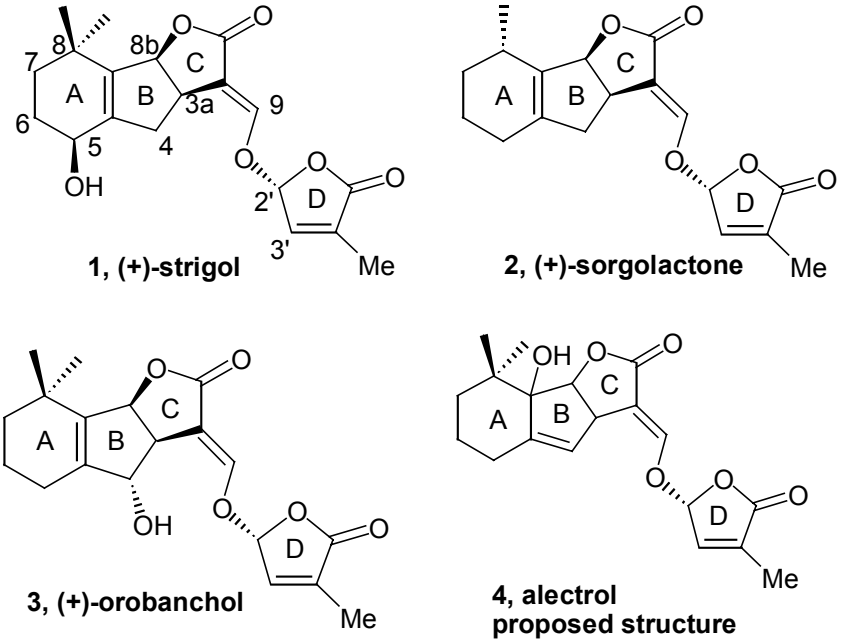
Important food crops, such as maize, sorghum, millet and rice, are host plants that can suffer enormously from these parasitic weeds, leading to considerable losses in crop yield, in some cases more than 50%. Thus far, four strigolactones have been isolated from the root exudates of host plants, namely (+)-strigol **1**[4], (+)-sorgolactone **2**[5], (+)-orobanchol **3**[6] and alectrol **4**[7] (Figure 1).The structures of the first three compounds have been secured by synthesis, while that of alectrol **4** is not established yet because the physical and spectral data of the synthesized compound having the proposed structure were not in agreement with those of the natural product.[8]

This chapter deals with an alternative synthesis of both (±)-strigol **1** and (±)-orobanchol **3**. (+)-Strigol **1**, being the first natural germination stimulant ever isolated[4], received much attention from

The content of this chapter was published: A. Reizelman and B. Zwanenburg, *Synthesis* **2000**, 1952-1955

synthetic chemists.[9-16] Nevertheless, there is still room for more efficient syntheses. (+)-Orobanchol **3** was prepared for the first time by Mori et al.[17] This stimulant was isolated by Yokota et al. from

Figure 1

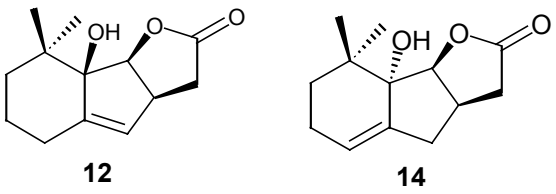


root exudates of red clover (*Trifolium pratense*).[6] As only a minute amount of stimulant could be obtained from this natural source, the structure elucidation was mainly based on GC-MS data. In fact, the position of the hydroxy group at C-4 could only be ascertained after completion of the synthesis and comparison with potential candidate structures, which were also prepared. The strategy followed by Mori et al. [17] is shown in scheme 1.[22]

The known lactone **5** (chapter 5) was oxidized to give a mixture of **6** and **7** by chromium trioxide in pyridine. The minor product **7** was then subjected to Luche reduction[18] to produce an epimeric mixture of **8a** and **8b**. The latter compound is the ABC-fragment of orobanchol **3**. After formylation and subsequent coupling with bromo butenolide **13** an epimeric mixture of **3a** and **3b** was obtained (epimer at C-2') which was separated by column chromatography. Though elegant, a shortcoming of this route is that orobanchol **3** is prepared from the minor oxidation product of **5**, namely **7**.

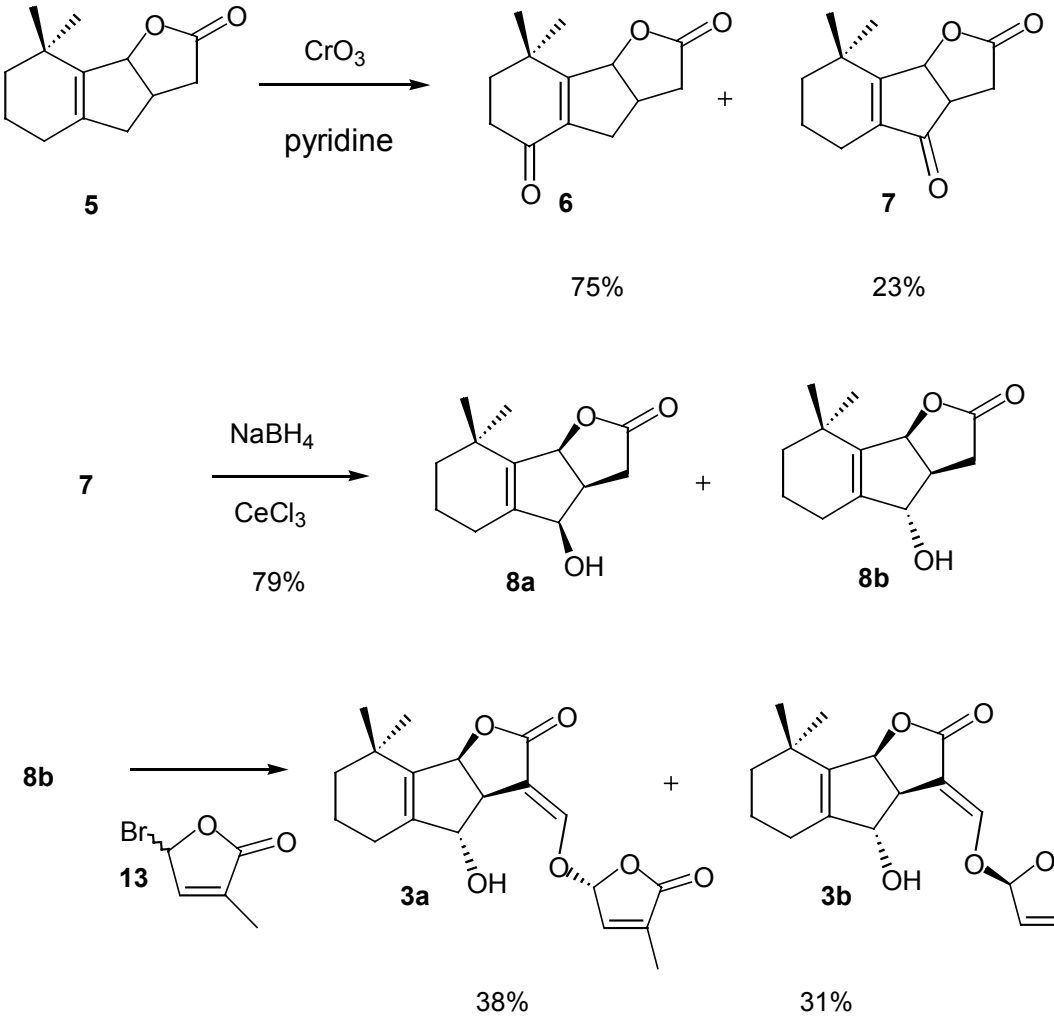
In the attempt, described in this chapter, to prepare alectrol with the proposed structure as shown in Chart 1, the efforts were directed towards the preparation of ABC-fragments **12** and **14** (Figure 2). When this work was in progress, compounds **12** and **14** were also prepared by Mori et al.[8]

Figure 2



12 **14**

Scheme 1



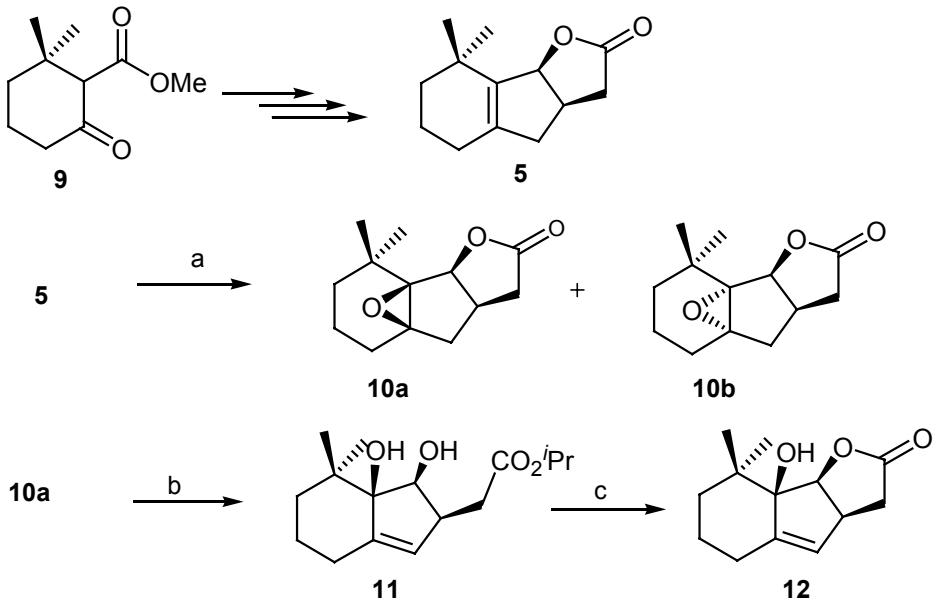
3.2 Results and Discussion

It was considered that an allylic rearrangement of **12** may lead to the ABC-skeleton of orobanchol **8** and that of **14** to ABC- skeleton of strigol **15**.

The tricyclic lactone **5** is an appropriate starting material for the synthesis of **12** and **14**, as is outlined in scheme 2.[22] The lactone **5** is readily available from β-keto ester **9** in 8 steps.[19] Epoxidation of **5** with *m*-chloroperbenzoic acid (*m*CPBA) gave a mixture of epoxides **10a** and **10b** in a ratio of 1:1.5 in a good yield, cf. ref.8. These isomeric epoxides could readily be separated by silica gel column chromatography. Compound **10a** was then subjected to treatment with aluminum isopropoxide in refluxing toluene to afford the transformation of the epoxide to the allylic alectrol unit. During this operation the lactone ring opened as well, to give *cis*-diol **11** in 66% yield.

The lactone ring could be readily reclosed to **12** by treatment with triethylamine in CH_2Cl_2. The crucial step, the allylic rearrangement, was accomplished by treatment with 3 equivalents of trifluoroacetic acid in CH_2Cl_2 and subsequent alkaline hydrolysis. A mixture of **8a** and **8b** was obtained in a ratio of 1:1 in a yield of 60% (Scheme 3). After chromatographic separation allylic alcohol **8b,** which has the correct relative stereochemistry of orobanchol, was coupled with bromo butenolide **13** in the standard fashion[14] to give the C-2' epimeric mixture of **3a** and **3b**. Separation by column chromatography afforded both epimers as single compounds.

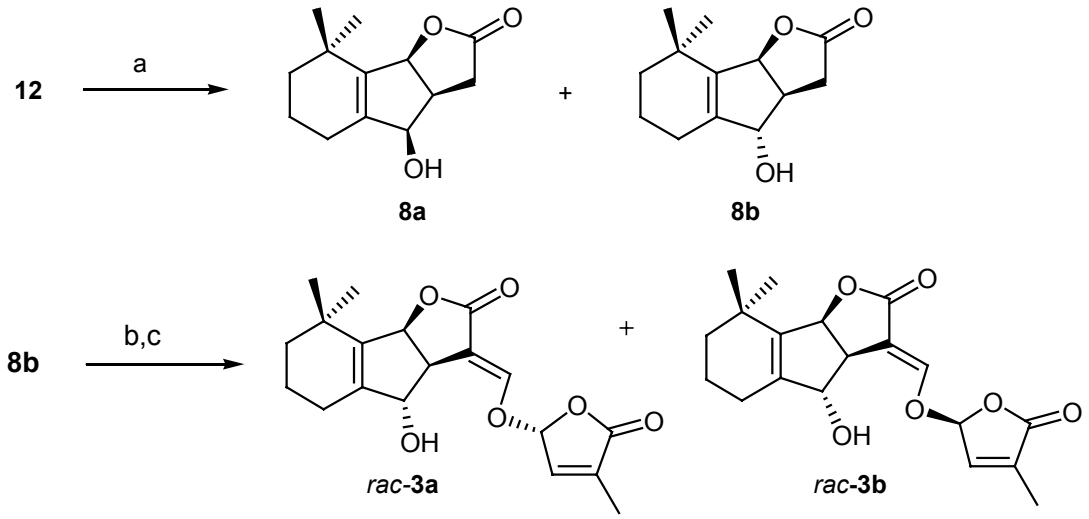
Scheme 2



Reagents: (a) *m*-CPBA, CH_2Cl_2 (80%). (1:1.5)- (b) $Al(OPri)_3$, toluene, reflux (66%).- (c) Et_3N, CH_2Cl_2, RT (90%).

In a similar manner the other epoxide, viz. **10b**, was converted to the hydroxy lactone **14** (Scheme 4). Allylic rearrangement of this material with trifluoroacetic acid and subsequent alkaline hydrolysis gave **15a**, which is the C-5 epimeric ABC-skeleton of strigol. It should be noted that only **15a** was obtained, no trace of **15b** was observed. This selectivity observed for the allylic rearrangement suggests that the gem-dimethyl in compound **14** hinders attack from the top face leading to the high stereoselctivity, whereas this effect is negligible in the reaction of compound **12**.

Scheme 3

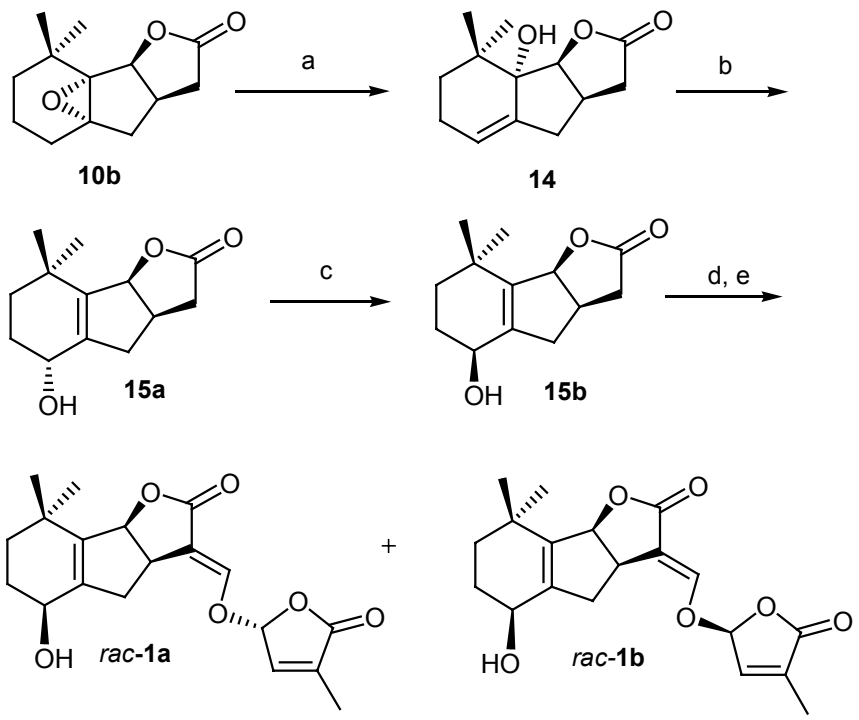


Reagents: (a) i. TFA, CH_2Cl_2, RT ii.K_2CO_3, MeOH (60%).(1:1)- (b) NaH, HCO_2Me, Et_2O.- (c) K_2CO_3, 4-bromo-2-methyl-2-buten-4-olide **13**, N-methylpyrrolidone; SiO_2 chromatog. (65%) α-isomer: β-isomer = 1:1

In order to obtain the strigol ABC-fragment with the correct stereochemistry at C-5, the configuration of the hydroxy group at that carbon atom has to be inverted. This operation was conveniently accomplished under Mitsunobu conditions.[20] The thus obtained product **15b** was then coupled with the chloro butenolide D-ring precursor[19] to give the epimeric strigol mixture consisting of **1a** and **1b**, which was separated by column chromatography. Separation of enantiomers can be achieved by preparative HPLC using a chiral column. This separation method has been described for strigol in chapter 2.[19]

In summary, this chapter describes the effective use of an allylic rearrangement for the synthesis of appropriately substituted ABC-fragment of strigolactones. In metabolic pathways leading to such strigolactones, similar allylic type rearrangements can be envisaged and maybe responsible for the formation of certain germination stimulants in nature.

Scheme 4



Reagents: (a) $Al(OPr^i)_3$, toluene, reflux (66%).- (b) i. TFA, CH_2Cl_2, RT ii. K_2CO_3, MeOH (90%).- (c) (i) $EtO_2CN{=}NCO_2Et$, Ph_3P, $PhCO_2H$, THF; ii) K_2CO_3, MeOH (70%).- (d) NaH, HCO_2Me, Et_2O.- (e) DBU, 4-chloro-2-methyl-2-buten-4-olide , CH_2Cl_2; silica gel chromatography. (62%) α-isomer: β-isomer = 1:1.

3.3 Experimental

General. ^1H NMR and ^{13}C NMR (300 MHz) spectra were recorded on a Bruker AC 300 spectrometer, using TMS as internal standard. Mps were measured with a Reichert thermopan microscope and are uncorrected. IR spectra were recorded on a Bio-Rad FTS-25 instrument. For mass spectra a double focusing VG7070E mass spectrometer was used. GC-MS spectra were run on a Varian Saturn 2 GC-MS ion-trap system. Separations were carried out on a fused-silica capillary column (DB-5, 30 m X 0.25 mm), helium was used as the carrier gas and electron impact (EI) was used as ionization mode. Elemental analyses were conducted on a Carlo Erba Instrument CHNSO EA 1108 element analyzer. For the determination of optical rotations a Perkin-Elmer 241 polarimeter was used. Thin layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F254 plates (0.25 mm). Column chromatography was performed with Merck Kieselgel 60.

All solvents were dried using standard methods. Reagents were obtained from commercial suppliers and were used without further purification.

10a and **10b**

These compounds were prepared according to a literature procedure[8].

Starting from the unsaturated lactone **9**[19], **10a** was obtained after chromatographic separation (silica gel; hexane/EtOAc; 3:1) as colorless crystals (50%), mp: 111-113 ^{0}C (hexane/CH_2Cl_2), Lit.[8]: mp: 110-111^{0}C and **10b** as colorless crystals (30%), mp: 72-74 ^{0}C (hexane/CH_2Cl_2), Lit.[8] mp: 61-62^{0}C. The structure of **10b** was analyzed by X-ray crystalography.[21] The spectral data of **10a** and **10b** were in a full accord with the reported data.[8]

(3a*R*,8a*R***,8b*R***)-8a-Hydroxy-8,8-dimethyl-3,3a,5,6,7,8,8a,8b-octahydro-2*H*-indeno[1,2-*b*]furan-2-one (12)**

A mixture of the epoxide **10a** (53 mg, 0.24 mmol) and aluminium isopropoxide (0.2 g, 0.95 mmol) in toluene (15 mL) was refluxed for 5 h. The cooled reaction mixture was then treated with HCl (2N) and extracted with ethyl acetate. The combined organic layers were washed with water and brine, and dried ($MgSO_4$). Removal of the solvent in vacuo gave crude diol **11**, obtained as yellowish oil (43 mg, 66%) after purification by column chromatography (silica gel; hexane/EtOAc; 2:1).The diol **11** was sufficiently pure for use in the subsequent step. A solution of **11** (43 mg, 152 mmol), triethylamine (0.021 mL, 152 mmol) in CH_2Cl_2 (10 mL) was stirred for 5 min at ambient temperature. The mixture was quenched with water and extracted with CH_2Cl_2 and the combined organic layers were washed with water and brine, and dried ($MgSO_4$). Removal of the solvent in vacuo gave **12** as colorless plates (40 mg, 90%), mp: 169-170^{0}C (ether), Lit.[8] mp: 169-170^{0}C. The spectral data of **12** were in a full accord with the reported data.[8]

(3a*S*,8a*S***,8b*R***)-8a-Hydroxy-8,8-dimethyl-3,3a,4,6,7,8,8a,8b-octahydro-2*H*-indeno[1,2-*b*]furan-2-one (14)**

These compounds were prepared from **10b** in a similar manner as described for diol **11**.

A mixture of the epoxide **10b** (327 mg, 1.47 mmol) and aluminium isopropoxide (30 mL, 1M solution of toluene, 20 equivalents) was refluxed for 3 days. The cooled reaction mixture was then treated with HCl (2N) and extracted with ether. The combined organic layers were washed with water and brine, and dried ($MgSO_4$). Removal of the solvent in vacuo gave **14**, as colorless crystals (219 mg, 66%) after purification by column chromatographic (silica gel; hexane/EtOAc; 3:1), mp 130-131^{0}C (hexane/EtOAc), Lit.[8] mp: 129-130^{0}C.

The spectral data of **14** were in a full accord with the reported data.[8]

(3a*R,4*R**,8b*R**)-4-Hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydro-2*H*-indeno[1,2-*b*]furan-2-one (8b)**

A solution of **12** (131 mg, 0.59 mol) and TFA (0.14 mL, 1.77 mmol) in CH_2Cl_2 (10 mL) was stirred for 1 h at ambient temperature. After TLC (hexane/EtOAc, 1:1) showed no trace of starting material, a saturated solution of sodium carbonate (10 mL) and MeOH (10 mL) were added and the resulting mixture was stirred for another 30 min. The solvents were removed in vacuo and the crude mixture was acidified with HCl (1N) and then extracted with EtOAc. The organic layer was washed with brine and dried ($MgSO_4$). Evaporation of the solvent and chromatographic separation (silica gel; hexane/EtOAc; 1:1) gave **8a** (38 mg, 29%) as colorless crystals, mp 76-78^{0}C (hexane/EtOAc), Lit.[17] mp: 79-80^{0}C and **8b** as colorless crystals (39 mg, 31%), mp: 85-90^{0}C. The spectral data of **8a** and **8b** were in a full accord with the reported data.[17]

(3a*S,5*S**,8b*R**)-5-Hydroxy-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydro-2*H*-indeno[1,2-*b*]furan-2-one (15b)**

Compound **15a** was obtained from **14** in the same manner as described for **8a** and **8b**. Product **15a** was obtained as an oil (90% yield from **14**) was sufficiently pure for the subsequent transformation.

To a stirred mixture of the alcohol **15a** (65 mg, 0.29 mmol), triphenylphosphine (129 mg, 0.44 mmol), benzoic acid (53 mg, 0.44 mmol) in THF (15 mL), diethyl azodicarboxylate (0.07 mL, 0.44 mmol) in THF (5 mL) was added. The mixture was stirred for 0.5 h and then concentrated under reduced pressure to give the crude benzoate (94 mg) after purification by column chromatography (silica gel; hexane/EtOAc; 1:1). The crude benzoate and K_2CO_3 (40 mg, 0.44 mmol) in MeOH (10 mL) was stirred for 5 h, acidified with HCl (1N) and then extracted with EtOAc. The organic layer was washed with brine and dried ($MgSO_4$). Evaporation of the solvent gave **15b** (45 mg, 70%) as colorless crystals after purification by column chromatography (silica gel; hexane/EtOAc; 1:1), mp: 146-148^{0}C (hexane/EtOAc), Lit[11] mp: 146^{0}C. The spectral data of **15b** were in a full accord with the reported data.[11,12]

3a and **3b**

In the same manner as described in ref.17, the lactone **8b** (60 mg, 0.27 mmol) was converted into **3a** (28 mg, 30%), colorless crystals, mp: 169-171^{0}C (hexane/EtOAc), Lit.[17] mp: 170-172^{0}C, and **3b** (33 mg, 35%) as colorless crystals, mp 198-200^{0}C (hexane/EtOAc), Lit.[17] mp: 200-201^{0}C.

The spectral data of **3a** and **3b** were in a full accord with the reported data[17].

1a and **1b**

Compound **15b** was formylated with ethyl formate and NaH as described by Brooks et al.[12] A solution of the formylated product (80 mg, 0.34 mmol) in CH_2Cl_2 (15 mL), DBU (0.52 mL, 0.37 mmol), chloro butenolide (55 mg, 41 mmol) in CH_2Cl_2 (5 mL) was added dropwise. The reaction stirred for 2 h at ambient temperature and then the solvent was evaporated in vacuo to give a crude mixture which were readily separated by column chromatography (silica gel; hexane/EtOAc; 3:1) to give **1a** obtained as a white solid, 52 mg (33%), mp 205-206^{o}C (hexane/EtOAc), Lit.[11] mp: 202-205^{o}C, and **1b** as a colorless crystals, 45 mg (29 %), mp 177-178^{0}C , Lit.[11] mp: 178-180^{0}C.
^{1}H NMR, ^{13}C NMR , MS , and IR where in full accord with the reported data.[11, 12]

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22 It should be noted that all structures shown in the schemes 1-3, chart 1 and 2, refer to racemic compound.

CHAPTER 4

An Efficient Enantioselective Synthesis of Strigolactones with a Palladium-Catalyzed Asymmetric Coupling as the Key Step

Abstract An efficient enantioselective methodology for the preparation of the strigolactones GR7, GR24 and Nijmegen-1, based on a palladium-catalyzed asymmetric transformation, was developed. The products were obtained in good yield and high optical purity. This methodology is an attractive alternative for installing the strereochemistry at C2' of the D-ring.

4.1 Introduction

Parasitic weeds belonging to the genera *Striga* and *Orobanche* spp. severely reduce yields of economically important crops in tropical areas of the eastern hemisphere.[1] Food crops, such as maize, sorghum, millet and rice, are host plant that can suffer enormously from these parasitic weeds, leading to considerable losses in crop yield, in some cases more than 50%. The parasitic process begins with the germination of the weed seeds, which is induced by a stimulant present in the root exudates of the host plant. Several naturally occurring germination stimulants, namely (+)-strigol[2] (Figure 1), (+)-sorgolactone[3], (+)-orobanchol[4] and alectrol[5] were isolated from host and non-host plants. Structure activity studies of the natural stimulants[6,7,8,9] and of their synthetic analogues GR7[10], GR24[11] and Nijmegen-1[12] (Figure 1) have revealed that the absolute configuration at the stereogenic centers of the stimulant is of great importance for the germination activity. For instance, the optical antipode of (+)-strigol has to be 500 times more concentrated to be as active as the natural isomer in the seeds of *Striga asiatica*[6].

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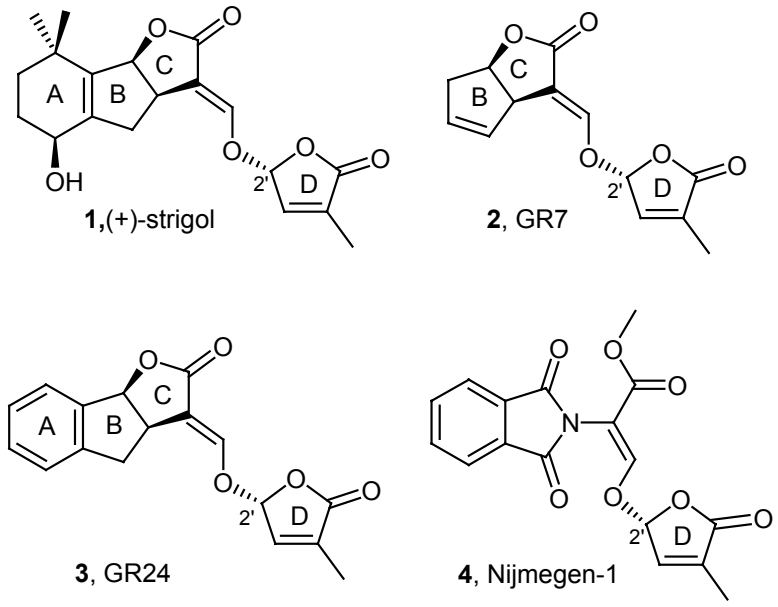
It may therefore be concluded that germination stimulants are highly selectively recognized by the parasitic seeds. Synthesis of strigol-type compounds is interesting for several reasons: *i.* to gain insight in the structure-activity relationships of these semiochemicals, *ii.* to provide the chemical basis for studying the biochemistry of the recognition and germination processes, and *iii.* to develop new methods for parasitic weed control[14].

Figure 1



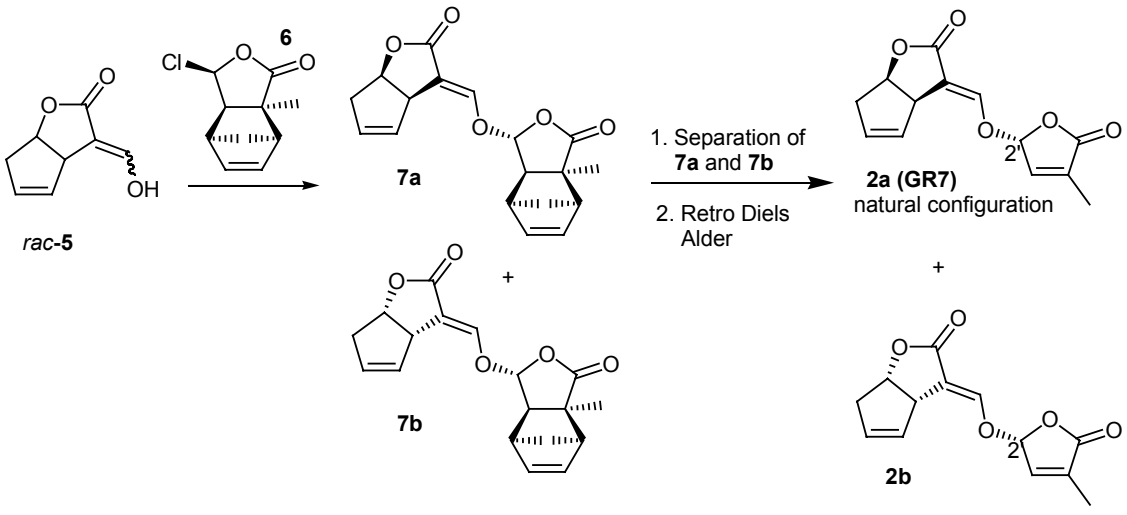
Several synthetic strigol analogs, commonly called strigolactones, have germination activity as high as the natural stimulants. The synthetic stimulant GR24 **(3)** is a very potent synthetic germination stimulant,[13a] which is used worldwide in parasitic weed research to stimulate germination and is a standard for comparing the activity of new germinating agents. Nijmegen-1[12] **(4)** is an attractive stimulant for *Striga* control by the suicidal germination approach.[14]

Over the years extensive efforts were made to prepare natural and synthetic stimulants in enantiopure form. The basic strategies reported hitherto for the synthesis of stereohomogenous strigolactones are: *i.* resolution of the racemic stimulant at the final stage by covalent coupling with a suitable resolving agent[15] or by chromatography on a chiral column,[6,9,16] *ii.* coupling of the enantiopure ABC fragment[10a,17–19] with the racemic

D-ring precursor and subsequent chromatographic separation of the obtained diastereomeric mixture, *iii.* coupling of the racemic ABC fragment with enantiopure D-ring precursor[10b,20,24] and subsequent separation of the resulting diastereomers, and *iv.* using starting materials from the chiral pool for the synthesis of enantiopure ABC fragment.[21–23]

Methodology of the type *iii* developed earlier in the Nijmegen laboratory involves the control of stereochemistry at C2' using the enantiopure D-ring synthon **6**[24] (Scheme 1). It should be emphasized that the absolute configuration at C2' is essential for biological activity. The Nijmegen methodology was successfully applied in the preparation of all eight stereoisomers of sorgolactone[8] as well as the synthetic stimulants GR7[10b], GR24[11], Nijmegen-1[12] and desmethylsorgolactone[25] in enantiopure form. Welzel *et al.* installed the correct stereochemistry at C2' in a highly selective manner using Winterfeldt's template as a chiral auxiliary.[26] Even though the products were obtained in high enantiopurity, preparation of the homochiral D-ring **6** or Welzel's D-ring precursor requires considerable synthetic effort. Furthermore, considerable material is lost during the removal of the auxiliary in the final step, resulting in yields of 50% at best (see also Chapter 1).

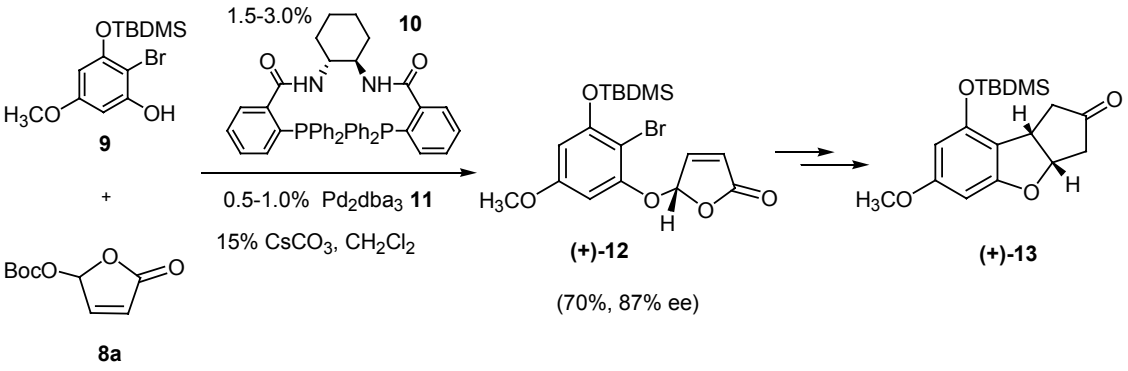
Scheme 1



An asymmetric transformation of racemic D-ring synthon with the racemic ABC-fragment would provide an attractive alternative to the existing strategies for the

synthesis of enantiopure strigolactones. For this purpose the palladium-mediated coupling methodology developed by Trost for the synthesis of lactone **13**, which constitutes an asymmetric formal synthesis of the aflatoxins[27] (Scheme 2), would be a conceivable approach for the asymmetric strigolactone synthesis. In this chapter, the results of such asymmetric coupling reactions of the D-ring precursor **8b** to the racemic ABC-part for the synthesis of the stimulants GR7, GR24 and Nijmegen-1, are reported.

Scheme 2



4.2 Results and Discussion

The coupling of the ABC fragment involves the reaction of an enol with a D-ring precursor having a suitable leaving group at the C-5 position. Initial attempts were carried out with the racemic enol **5**[10], which constitutes the ABC-part of the stimulant GR7. Coupling with racemic D-ring precursor **8b**[28] containing a -OBoc as leaving group with the achiral ligand 1,2-bis(diphenylphosphino)ethane (9%) in the presence of Pd_2dba_3 (3 mol %) **11** resulted in a mixture of racemic coupling products *rac*-**2a** and *rac*-**2b** in a ratio of 1:1.5 in a 90% yield after 1 hour. These diastereomers could be readily separated by column chromatography. These coupling experiments were performed with ca. two equivalents of the D-ring precursor, although one equivalent would suffice. Trost[27] also used two equivalents of the electrophilic reagents in his coupling reactions. Having

demonstrated the feasibility of the Pd-mediated coupling, the use of a chiral ligand, *viz.* Trost ligand (*R,R*)-**10**[29] in the presence of the Pd_2dba_3 was considered next (Scheme 3).

Scheme 3

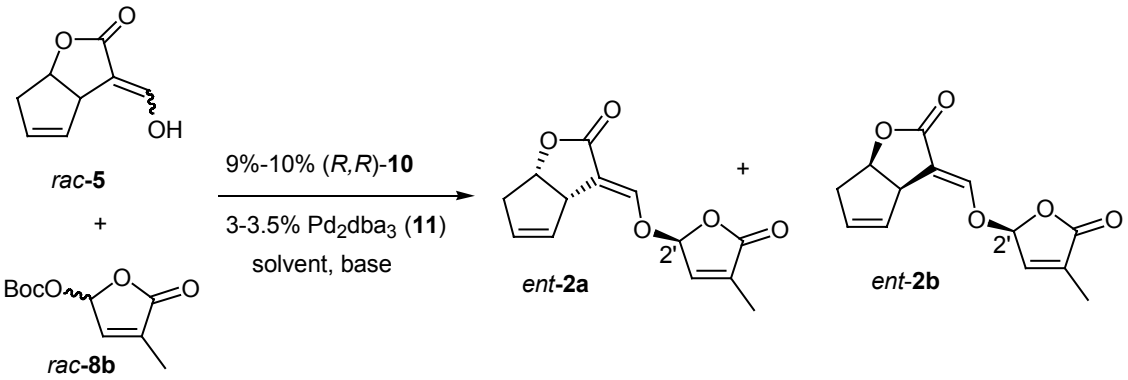


*Table 1. Asymmetric coupling of rac-**5** with rac-**8b*** [a]

Entry	Solvent/base	Ligand	Absolute configuration at C2'[b]	Yield[c] (%)	Product (*ee*)[d]	
1	THF	*(R,R)*-**10**	*S*	90	*ent*-**2a** (78)	*ent*-**2b** (86)
2	THF/ Et_3N	*(R,R)*-**10**	*S*	89	*ent*-**2a** (75)	*ent*-**2b** (84)
3	CH_2Cl_2/ Et_3N	*(R,R)*-**10**	*S*	74	*ent*-**2a** (88)	*ent*-**2b** (92)
4[e]	CH_2Cl_2/ Et_3N	*(R,R)*-**10**	*S*	67	*ent*-**2a** (96)	*ent*-**2b** (98)
5	CH_2Cl_2/ Et_3N	*(S,S)*-**10**	*R*	88	**2a** (90)	**2b** (98)
6[e]	CH_2Cl_2/ Et_3N	*(S,S)*-**10**	*R*	75	**2a** (93)	**2b** (94)

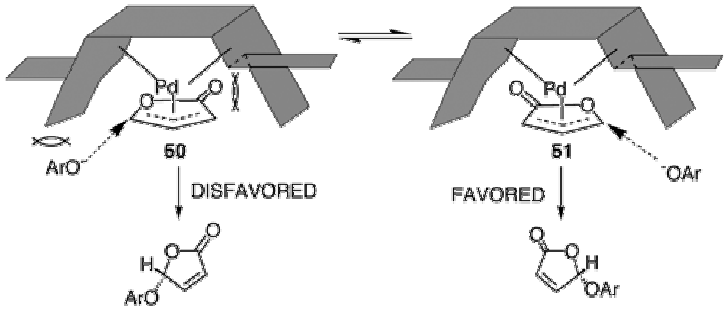
[a] Unless otherwise stated all reactions were carried out at room temperature, using 3.0-3.5% $Pd_2(dba)_3$,$CHCl_3$ and 9-10% ligand (*R,R*)-**10** or **(***S,S***)-10** The ratio of **5**, **8b** and triethylamine amounts to 1:2:1. [b] The absolute configurations were determined by comparison of their optical rotation with those reported[10]. [c] Combined yield of **2a** and **2b,** or of *ent*-**2a** and *ent*-**2b**. The diasteriomeric ratio is 1:1.5 in both cases. [d] Determined by HPLC on a Chiralcel OD column with hexane/EtOH or hexane/2-propanol, 1 mL/min, 254 nm. [e] The temperature employed was –10°C to room temperature.

Under similar conditions, a mixture of *ent*-**2a** and *ent*-**2b** with enantiopurities of 78% and 86%, respectively, was obtained in 90% yield (table 1, entry 1). Addition of a tertiary amine base, Et_3N, to facilitate the nucleophilic reaction of the enol had no significant effect on the enantioselectivity (entry 2). Changing the solvent from THF to CH_2Cl_2, still using Et_3N as a base, improved the ee of the coupling products to 88% and 92%,

respectively (entry 3). Lowering the temperature resulted in an excellent ee's of 96% and 98%, respectively (entry 4). The absolute configuration at C2' of *ent*-**2a** and *ent*-**2b** was assigned as (*S*) by comparing the optical rotations of both coupling products with those reported.[10] The resulting stereochemistry at C2' is opposite to that of naturally occurring strigol. In order to obtain the diastereomers with the natural configuration at C2', the ligand (*S,S*)-**10** was used which resulted in the expected diastereomers of GR7 with high ee's (entries 5 and 6).

In the coupling reactions using the chiral ligand two equivalents of the D-ring precursor *rac*-**8b** were also employed, although one equivalent theoretically should be sufficient. Supposedly, the reaction rate is favorably influenced by the excess of this reagent. Mechanistically, the D-ring precursor *rac*-**8b** forms a π-allyl complex of which one face is shielded by the chiral ligand (Figure 2). The OBoc group serves as the leaving group in the formation of the Pd-complex. It has been demonstrated by Trost that the face selectivity of such chiral Pd-complexes can be very high[27,29] (Figure 2).

Figure 2



Since all possible stereoisomers of stimulant GR7 were successfully obtained using the Pd-catalyzed coupling, the methodology was next extended to the preparation of the four diastereomers of stimulant GR24 **(3)** and both enantiomers of Nijmegen-1 **(4)** (table 2,

scheme 3 and 4). Under the conditions indicated in scheme 3, the enol of racemic lactone **14**[11] was coupled to give stereoisomers **3a** and **3b**, when chiral ligand (*S,S*)-**10** was used (Scheme 4), with ee's of 90% and 100%, respectively. Lowering the temperature or the catalyst loading of Pd_2dba_3 to 2% did not improve this optical yield. However, crystallization improved the enantiopurities of **3a** and **3b** from 88% and 93% to 90% and 100%, respectively. When the antipodal catalyst (*R,R*)-**10** was used *ent*-**3a** and *ent*-**3b** were similarly obtained in high optical yields (table 2, entry 2).

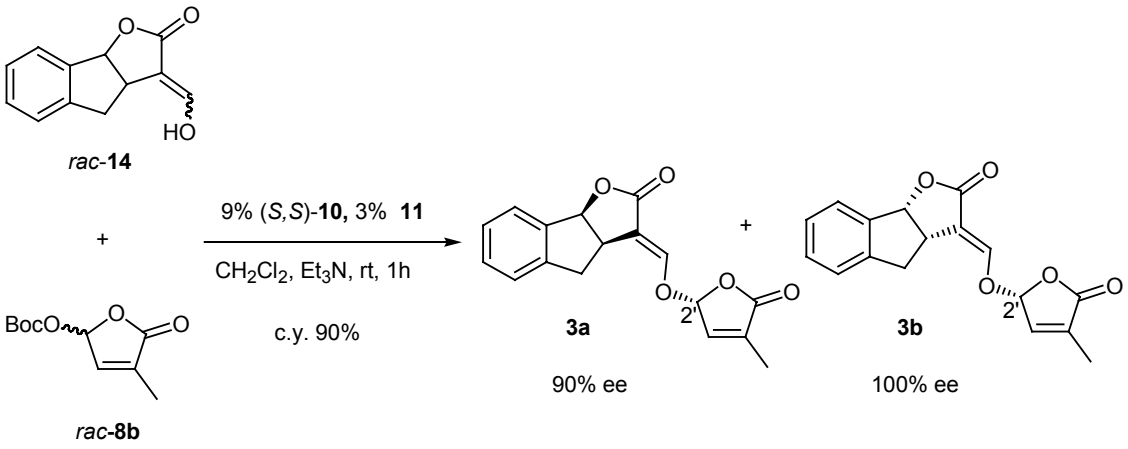
Coupling of **15,**[12] which is the basic fragment of Nijmegen-1, favors the enantiomer with the natural configuration at C2' when (*S,S*)-**10** was used as the chiral catalyst, while the antipode *ent*-**4** was obtained by employing (*R,R*)-**10** as the ligand in the Pd-catalyzed coupling (table 2, entry 3 and 4).

*Table 2. Asymmetric coupling of rac-**14** and rac-**15** with rac-**8b**[a]*

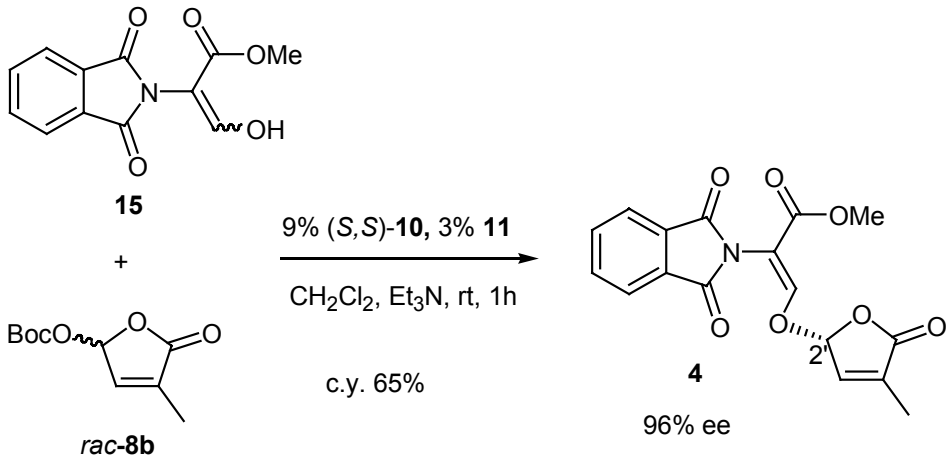
Entry	Enol	Ligand	Absolute configuration at C2'[b]	Yield (%)	Products (ee %)[d]
1	**14**	(*S,S*)-**10**	*R*	90 [c]	**3a** (90) [e] **3b** (100)[e]
2	**14**	(*R,R*)-**10**	*S*	85 [c]	*ent*-**3a** (94) *ent*-**3b** (100)
3	**15**	(*S,S*)-**10**	*R*	65	**4** (96)
4	**15**	(*R,R*)-**10**	*S*	60	*ent*-**4** (97)

[a] All reactions were carried out at room temperature in dichloromethane as the solvent, using 3.0% $Pd_2(dba)_3$, $CHCl_3$ and 9% (*R,R*)-**10** or (*S,S*)-**10**. The ratio of **14** or **15**, **8b** and Et_3N amounts to 1:2:1.[b] The absolute configurations were determined by comparison of their rotation with the reported values.[11,12]
[c] Combined yield of both diastereomers obtained in a ratio of 1:1.4. [d] Determined by HPLC on Chiralcel-OD® column with hexane/EtOH mixtures. [e] After recrystallization from hexane/ethyl acetate mixture.

Scheme 4



Scheme 5



In conclusion, a short and efficient methodology for the asymmetric synthesis of all possible stereoisomers of the strigolactones GR7, GR24 and Nijmegen-1 has been developed. The key step is the palladium-catalyzed asymmetric transformation of lactones **5**, **14**, **15**, with the D-ring precursor *rac*-**8b**. This methodology is an attractive alternative to the existing routes which in most cases are more laborious and result in lower yields. This also provides the first reported example of a palladium catalyzed *O*-alkylation using enols. On the basis of the successful coupling in the syntheses of three

stimulants, it is likely that this methodology will be extended to practically any synthesis of a strigolactone, including the naturally occurring ones.

4.3 Experimental

General: Lactones *rac*-**5**[10], *rac*-**14**[11], *rac*-**15**[12] were prepared according to procedures described in the literature. All reagents were obtained commercially and were used without purification. All reactions were carried out under an inert atmosphere (Argon). Tetrahydrofuran was distilled from sodium benzophenone ketyl. Dichloromethane was distilled from calcium hydride. Triethylamine was distilled from KOH and stored over molecular sieves. Column chromatography was carried out on E. Merck silica gel 60 (60-75 mesh) using hexane/ethyl acetate mixture. All enantiomeric excesses were determined using the column Chiralcell-OD®, 4.6 mm X 25 cm; solvent: hexane/EtOH or hexane/2-propanol, flow rate: 1 mL/min; detector: 254 nm; temperature: room temperature.

General Procedure for the coupling

To a test tube containing *rac*-**8b** (0.257 mmol, 2 equiv.), **11** (0.004 mmol, 3%) and ligand (S,S)-**10** (0.012 mmol, 9%), CH_2Cl_2 (1 mL, dry and oxygen free) was added. The resulting purple reaction was stirred for 20 min., resulting a clear yellow solution. The yellow reaction mixture was then treated with Et_3N (0.129 mmol, 1equiv.) and *rac*-**14** (0.129 mmol, 1 equiv.) and subsequently stirred at room temperature until TLC (hexane/EtOAc 1:1) showed no trace of starting material any more (1 hr). The resulting yellow reaction mixture was absorbed on silica and chromatographed (hexane/ethyl acetate = 1/1) to afford the product as a colorless solid (67% yield). The product was subjected to determination of the enantiomeric purity using column Chiralcel-OD®, solvent: hexane/EtOH, flow rate: 1 mL/min.

* Note: The coupling reactions can also be performed on a gram scale.

Ent-**2a** and *ent*-**2b**: To a test tube containing *rac*-**8b** (35 mg, 0.160 mmol), **11** (3 mg, 0.003 mmol) and ligand (*R,R*)-**10** (6 mg, 0.009 mmol), CH_2Cl_2 (1 mL, dry and oxygen free) was added. The resulting purple reaction was stirred for 30 min. at -10^0C, resulting

a clear yellow solution. To this reaction mixture Et_3N (12 µL, 0.086 mmol) and *rac*-**5** (13 mg, 0.086 mmol) was added. The reaction mixture was allowed to warm up to room temperature. After TLC (hexane/EtOAc 1:1) showed no trace of starting material (3 h) any more, the crude mixture was absorbed on silica and chromatographed (hexane/ethyl acetate 1:1) to afford the products in a total yield of 65%. The diastereomeric ratio of *ent*-**2a** and *ent*-**2b** was 1:1.5, respectively (see table 1,entry 4).

Ent-**2a:** $[\alpha]_D$ -330^0 (c 1.00, C_2HCl_2) [ref.[10] $[\alpha]_D$ -341^0 (c 1.03, C_2HCl_2)].
Chiral HPLC analysis; OD column (6:4 hexane: 2-Propanol, 1 mL/min): t_R = 8.5 min (2 %) and 9.6 min (95%.) The enantiomeric purity of *ent*-**2a** was estimated to be 96% **.**

Ent-**2b;** $[\alpha]_D$ -165^0 (c 1.03, C_2HCl_2) [ref.[10] $[\alpha]_D$ -179^0 (c 1.44, C_2HCl_2]).
Chiral HPLC analysis; OD column, (8:2 hexane:EtOH, 1 mL/min) t_R = 9.3 (95%) and 11.1 min (1%). The enantiomeric purity of *ent*-**2b** was estimated to be 98%.
Their spectral data (^{1}HNMR, ^{13}C NMR and IR) were in full accord with those reported in the literature.[10]

Compounds 2a and **2b**: obtained as described for *ent*-**2a** and *ent*-**2b**, using the (*S,S*)-**10** catalyst. Purification by chromatography (SiO_2, hexane/ethyl acetate 1:1) gave the products in a total yield of 75% and in a diastereomeric ratio of 1:1.5 (see table 1,entry 6).

2a: $[\alpha]_D$ $+320^0$ (c 1.1, C_2HCl_2) [ref.[10] $[\alpha]_D$ $+339^0$ (c 1.06, C_2HCl_2)].
Chiral HPLC analysis; OD column (8:2 hexane:EtOH, 1 mL/min) t_R = 11.5 min (5%) and 12.5 min (95%). The enantiomeric purity of **2a** was estimated to be 90%.

2b: $[\alpha]_D$ $+165^0$ (c 1.40, C_2HCl_2) [ref.[10] $[\alpha]_D$ $+174^0$ (c 1.44, C_2HCl_2)].
Chiral HPLC analysis; OD column t_R = 9.26 min (3.6 %) and 11.0 min (95%). The enantiomeric purity of **2b** was estimated to be 93% .
Their spectral data (^{1}HNMR, ^{13}C NMR and IR) and were in full accord with those reported in the literature.[10]

Compounds 3a and **3b:** These compounds were synthesized according to the general procedure starting form *rac*-**14** (33 mg, 0.163 mmol) using the (*S,S*)-**10** catalyst. The reaction was completed after 1h. Purification by chromatography (SiO_2, hexane/ethyl acetate 1:1) gave the products in a total yield of 90%. The diastereomeric ratio was 1:1.4 (see table 2,entry 2). Their spectral data (^{1}HNMR, ^{13}C NMR and IR) were in full accord with those reported in the literature.[11]

3a: $[\alpha]_D$ $+420^0$ (c 0.2, $CHCl_3$) [ref.[11] $[\alpha]_D$ $+436^0$ (c 0.25, $CHCl_3$)].
Chiral HPLC analysis; OD column (7:3-8:2 gradient hexane: EtOH, 1 mL/min) t_R = 10.6 min (3 %) and 12.8 min (93.4 %). The enantiomeric purity of **3a** was estimated to be 90% after single crystallization (hexane/ethyl acetate).
3b: $[\alpha]_D$ -274^0 (c 0.2, $CHCl_3$) [ref.[11] $[\alpha]_D$ -272^0 (c 0.2, $CHCl_3$)].Chiral HPLC analysis; OD column (7:3 hexane/EtOH, 1 mL/min) t_R = 9.3 min (not detected by the HPLC) and 11.4 (100%) min. The enantiomeric purity of **3b** was estimated to be 100% .

Ent-**3a** and *ent*-**3b:** These compounds were synthesized according to the general procedure starting form *rac*-**14** (49 mg, 242 mmol) using the (*R,R*)-**10** catalyst. The reaction was completed after 1 h. Purification by chromatography (SiO_2, hexane/ethyl acetate 1:1) gave the products in a total yield of 90%. The diastereomeric ratio was 1:1.4, (see table 2,entry 1).

Ent-**3a:** $[\alpha]_D$ -436^0 (c 0.25, $CHCl_3$) [ref.[11] $[\alpha]_D$ -446^0 (c 0.25, $CHCl_3$)].
Chiral HPLC analysis; OD column (7:3 hexane: EtOH, 1 mL/min) t_R = 10.3 min (94.7 %) and 12.06 min (3 %). The enantiomeric purity of *ent*-**3a** was estimated to be 93%.

Ent-**3b:** $[\alpha]_D$ $+270^0$ (c 0.2, $CHCl_3$) **[**ref.[11]$[\alpha]_D$ $+273^0$ (c 0.2, $CHCl_3$)].
Chiral HPLC analysis; OD column (7:3 hexane:EtOH, 1 mL/min) t_R = 8.72 (100%) and 10.1 min (not detected by the HPLC). The enantiomeric purity of *ent*-**3b** was estimated to be 100%.
Their spectral data (^{1}HNMR, ^{13}C NMR and IR) were in full accord with those reported in the literature.[11]

Compound 4: This compound was synthesized according to the general procedure starting from **15** (32 mg, 0.129 mmol) using the (*S,S*)-**10** catalyst. The reaction was competed after 1h. Purification by chromatography (SiO_2, hexane/ethyl acetate 1:1) gave the product in a total yield of 65% yield. (see table 2,entry 3).

4: $[\alpha]_D$ $+118^0$ (c 0.1, CH_2Cl_2) [ref.[12] $[\alpha]_D$ $+124^0$ (c 0.15, CH_2Cl_2)].
Chiral HPLC analysis; OD column (7:3 hexane:EtOH, 1 mL/min) t_R = 8.0 min (93 %) and 11.7 min (1.7%). The enantiomeric purity of **4** was estimated to be 96% .

Compound *ent*-**4:** This compound was synthesized according to the general procedure starting form **15** (33 mg, 0.129 mmol) using the (*R,R*)-**10** catalyst. The reaction was completed after 1h. Purification by chromatography (SiO_2, hexane/ethyl acetate 1:1) gave the product in a total yield of 65% yield (see table 2,entry 4).

Ent-**4:** $[\alpha]_D$ -120^0 (c 0.1, CH_2Cl_2) [ref.[12] $[\alpha]_D$ -128^0 (c 0.15, CH_2Cl_2)].
Chiral HPLC analysis; OD column (7:3 hexane:EtOH, 1 mL/min) t_R = 11.9 min (94.5 %) and 7.4 (1.4 %) min. The enantiomeric purity of *ent*-**4** was estimated to be 97%.
Their spectral data (^{1}HNMR, ^{13}C NMR and IR) were in full accord with those reported in ref[12].

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CHAPTER 5

Synthesis and Bioactivity of Labelled Germination Stimulants for the Isolation and Identification of the Strigolactone Receptor

Abstract Strigolactones are highly potent germination stimulants for seeds of the parasitic weeds *Striga* and *Orobanche* spp. The induction of seed germination is thought to proceed via a receptor-mediated mechanism. Isolation and purification of the strigolactone receptor by affinity chromatography using immobilized avidin or streptavidin requires a biotin labelled strigolactone analogue. For this purpose biotin has been attached, directly as well as indirectly, via a hydrophilic linker to the amino function of optically active amino-GR24. Using the same amino substituted synthetic stimulant GR24, labelled stimulants have been prepared which may be suitable for the identification of the receptor by means of fluorescence correlation spectroscopy, scanning force microscopy or photoaffinity techniques. Bioassays of the labelled stimulants reveal that the germination activity on seeds of *Striga hermonthica* is retained.

5.1 Introduction

Parasitic weeds belonging to the genera *Striga* and *Orobanche* severely reduce yields of economically important crops worldwide, especially in tropical and sub-tropical areas.[1] Food crops, such as maize, sorghum, millet and rice, are host plants that can suffer enormously from these parasitic weeds, leading to considerable losses in crop yield, in some cases more than 50%. Several naturally occurring germination stimulants, viz. (+)-strigol[2] **1**, (+)-sorgolactone[3] **2**, (+)-orobanchol[4] and alectrol[5] were isolated from host and non-host plants (Figure 1). Structure-activity studies of the natural stimulants[6,7,8,9] and

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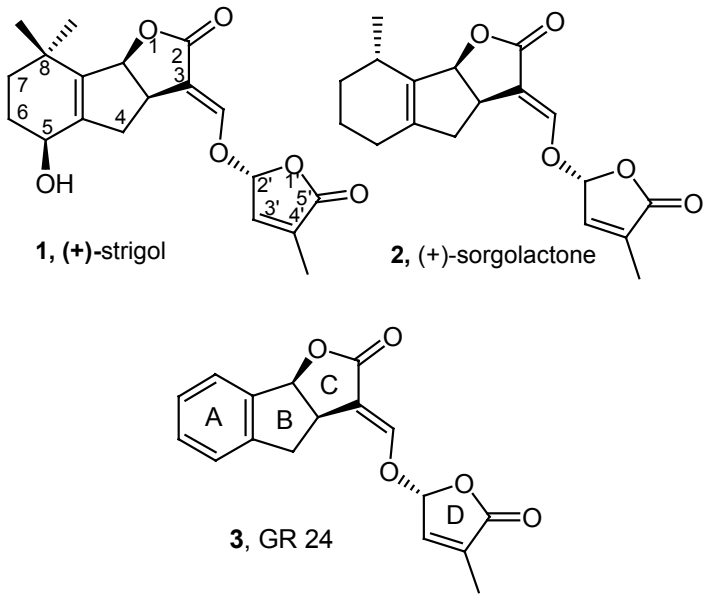
The chemistry presented in the schemes **6, 7** and **8** was also described in the thesis of Dr. S.C.M. Wigchert, University of Nijmegen, **1999**.

their synthetic analogues GR7[10] and GR24[11] and Nijmegen-1[12] revealed that: *i.* Strigolactones are active at a low concentration (10^{-8}-10^{-12}M), *ii*. The bioactiphore resides in the CD-part of these molecules[13,14], *iii.* The absolute configuration at the CD-moiety is of great importance for the seed germination activity.[8,15] These results strongly suggest that the induction of *Striga* and *Orobanche* seed germination proceeds via a receptor-mediated mechanism.[8,13,14] So far, nothing is known about the protein structure of this hypothesized receptor, nor of its localization within the seeds. Detailed knowledge of the receptor protein would provide insight in the initial stages of the germination process of these seeds and would enable the design of a perfectly fitting substrate that might be used to control parasitic weeds pests by the suicidal germination approach.[16,17,20]

Figure 1



Affinity chromatography is a widely applied separation technique for the isolation of molecules of biological interest. One popular application involves tagging an antibody or a ligand with biotin and then attaching it to a solid support to which the protein avidin/streptavidin has been linked. Once the antibody or ligand has been attached to the

solid support, it can then be used as a stationary phase for the purification of the antigen in the case of an antibody, or of the receptor in case of a ligand. The use of biotin and avidin/streptavidin to link to the solid support depends on the high affinity of avidin or streptavidin for biotin (K_d=10^{-14}-10^{-15}M).[18] To avoid steric hindrance and to allow an optimal association of biotin to streptavidin/avidin usually an extended spacer is incorporated. The ethylene glycol polymers (PEG) have been examined and have the required properties as linkers in biological systems because of their solubility and stability in aqueous solution at physiological pH.

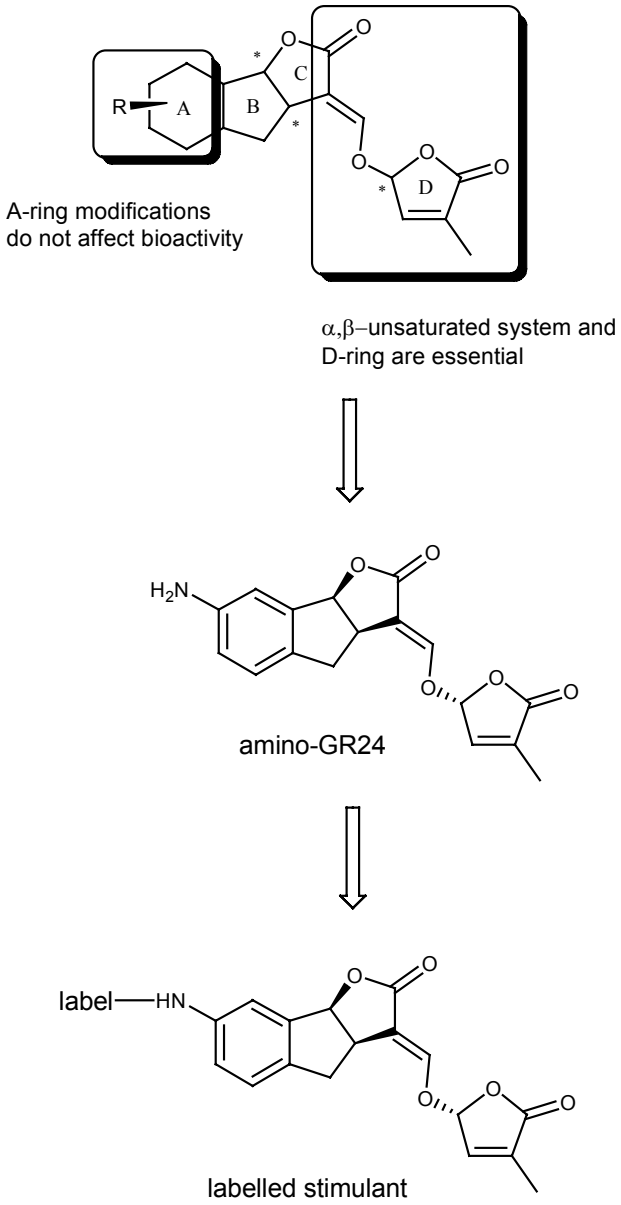
Isolation and purification of the strigolactones receptor by affinity chromatography through immobilized avidin/streptavidin required a biotin labeled strigolactone analogue. A suitable synthetic stimulant for labelling is GR24 **3** (Figure 1) which exhibits high activity towards *Striga* and *Orobanche* seeds.[19] It should be emphasized that the CD-part is essential for biological activity. The A-ring is in a remote position with respect to the bioactiphore and therefore, various substituents have been introduced in this ring without seriously effecting the bioactivity.[20] This then leads to the strategy outlined in Fig. 2 for the synthesis of appropriately labelled stimulants on the basis of GR24.

In fact, the synthesis of some biologically active, labelled strigolactone analogues suitable, at least in principle, for the identification of the strigolactone receptor, has been reported previously from the Nijmegen laboratory.[20,34] In order to achieve optimal binding to the receptor, the stereochemistry of the stimulant moiety should be the same as that of natural strigolactones. This chapter describes the synthesis of optically active amino-GR 24 and the subsequent attachment of biotin directly as well as via a hydrophilic spacer (PEG).

Several other methods can be considered to gather information about the protein receptor, *e.g*. Fluorescence Correlation Spectroscopy (FCS),[21,22] Scanning Force Microscopy (SFM)[23] and Photoaffinity labelling (PAL).[24,25,26] In FCS the labelled substrate is exposed to a focused laser beam whereby fluorescent molecules will be excited to give a burst of photons that can be detected. FCS is attractive for the current problem as it is highly sensitive, provided efficient fluorescent labels are used. This chapter describes the preparation of a strigolactone analogue with such a fluorescent tag, using the strategy shown in figure 1. In SFM the ligand, which responds to a receptor, is attached to a gold SFM-tip using a linker with a free SH group. A strigolactone analogue containing such a

SFM linker has been prepared.[34] PAL requires the attachment of a photoreactive unit to the ligand, *e.g.* an azido group, allowing the generation of a very reactive, short-lived intermediate upon irradiation, which will immediately form a covalent bond with the molecule in its nearest vicinity, *i.e.* the protein receptor.

Figure 2



A strigolactone analogue containing a photoreactive tag has been prepared from amino-GR24.[34b] In all strigolactone analogues mentioned above a suitable substituent has been introduced at the A-ring of GR24. In order to serve their purpose in the isolation or identification of the strigolactone receptor these analogues must have germination activity. Thus, bioassays of these compounds with seeds of *Striga hermonthica* have been performed.

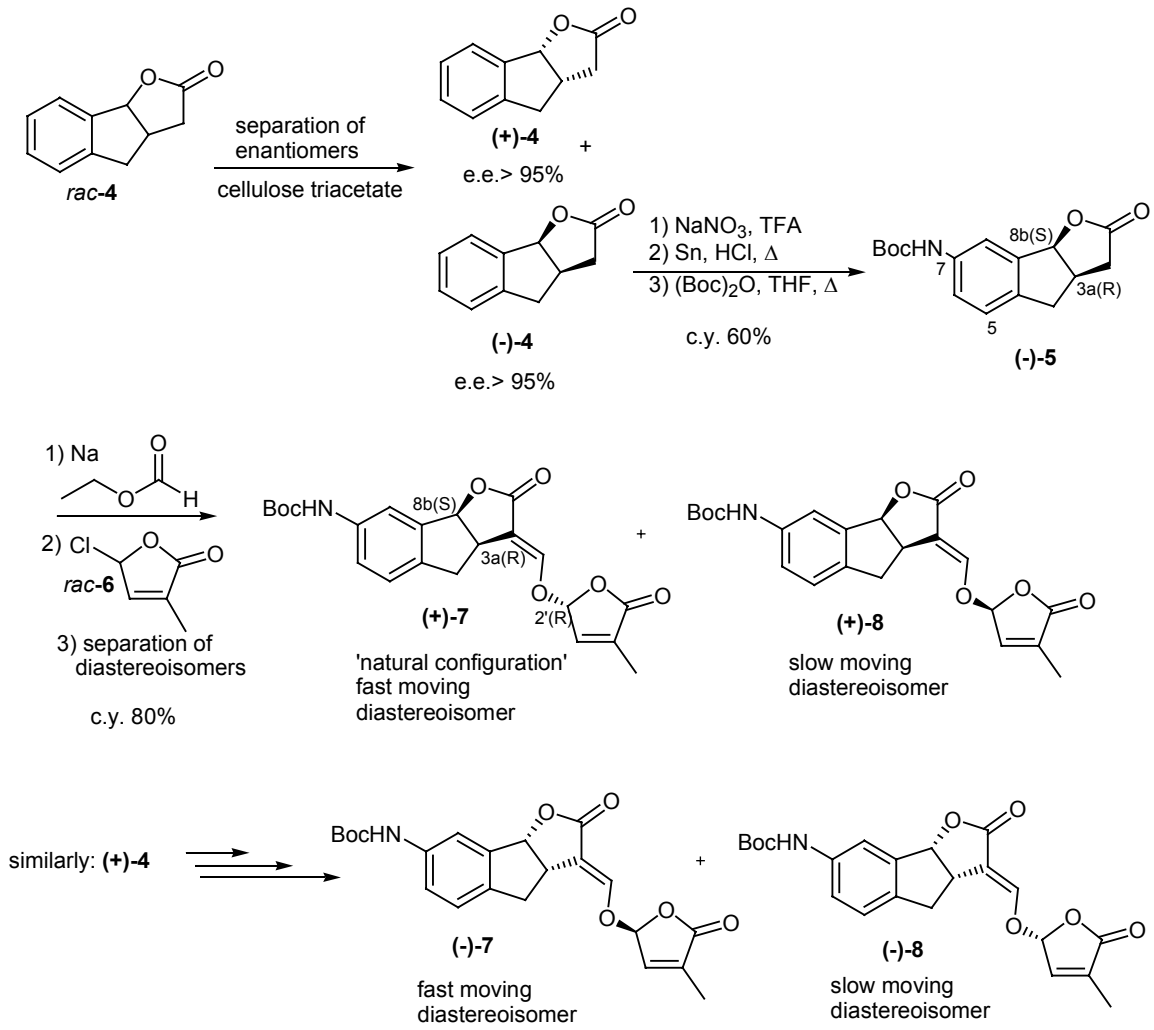
5.2 Results and discussion

Synthesis

Enantiopure amino-GR24 (+)-**7**, having the same configuration as the natural strigolactones, is a key compound in the synthesis of labelled strigolactone analogues. The synthesis involves the coupling of chlorobutenolide[27] *rac*-**6** to enantiopure amino-ABC-lactone (-)-**5** as depicted in Scheme 1. In order to obtain the desired optically pure (-)-**5**, racemic tricyclic lactone *rac*-**4** was chromatographically resolved using cellulose triacetate, as described previously.[19b] Nitration of (-)-**4** followed by reduction of the nitro group to an amine substituent was performed using tin and hydrochloric acid according to the method of Thuring *et al*[20]. The nitration also gave some C_5-nitro product (ratio C_7-NO_2 : C_5-NO_2 = 9:1). The preference for nitration at C_7 can be explained by the the higher electron density at that carbon atom. Prior to formylation and coupling with chlorobutenolide *rac*-**6**, the amino substituent was protected with a *tert*-butyloxycarbonyl (Boc) group. Formylation of **(-)-5** employing sodium in ethyl formate as the solvent, followed by reaction of the resulting sodium enolate with *rac*-**6** in THF gave protected enantiopure amino-GR24 diastereoisomers (+)-**7** and (+)-**8**. These two diastereoisomers could readily be separated by chromatography on silica gel. In a similar manner**,** enantiopure amino-GR24 diastereomers (-)-**7** and (-)-**8** were synthesized from tricyclic lactone (+)-**5**. The stereochemistry of these four diastereoisomers of amino-GR24 was secured as follows. The absolute stereochemistry of the tricyclic lactones **(-)-4** and **(-)-5** is correlated with GR24 having the natural configuration at C_{8b} and C_{3a}, namely *S* and *R*, respectively.[19b] CD spectra can now be used to determine the stereochemistry at C2' in (+)-**7** and (-)-**7**. It has been demonstrated by Welzel *et al.*,[28,29] that the sign of the Cotton effect at 270 nm is directly correlated with the stereochemistry at C2', namely, a negative Cotton effect at this

wavelength corresponds with the C2'(*R*) configuration and a positive sign with the C2'(*S*) configuration.

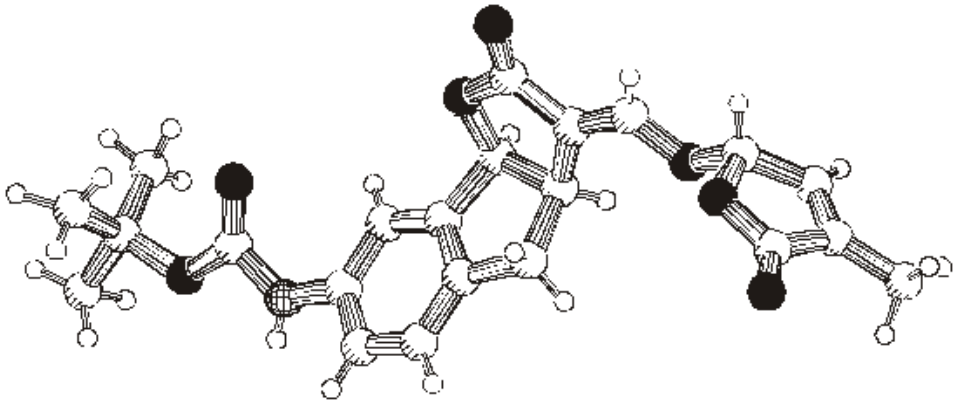
Scheme 1



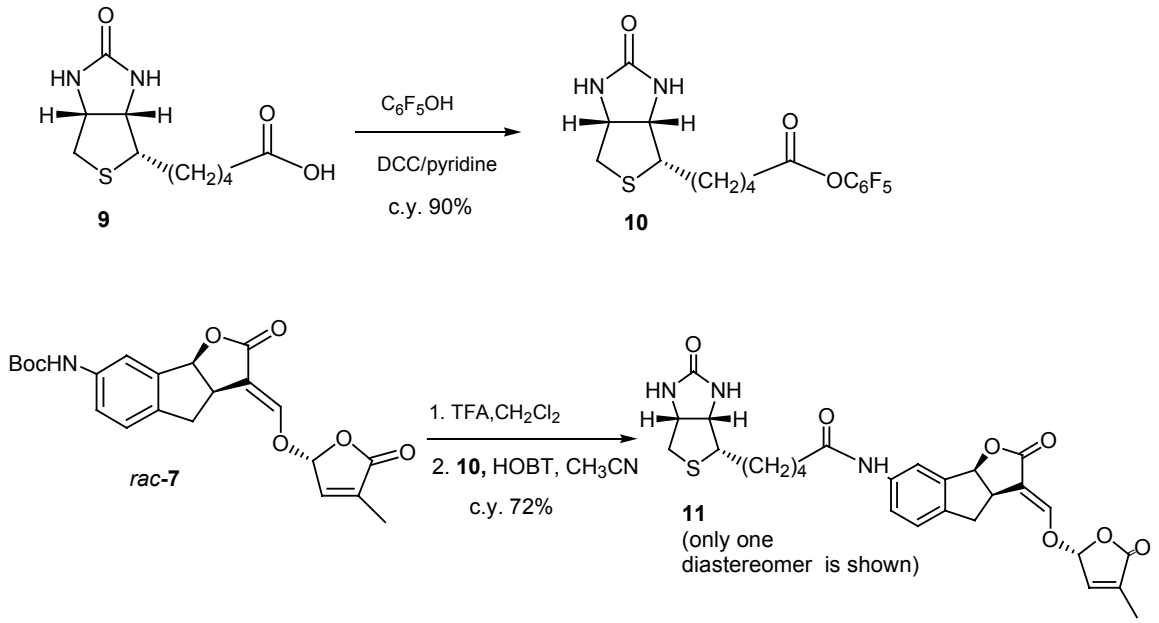
This rule has proven its value in establishing the stereochemistry in several strigolactones, especially GR28[30], desmethyl sorgolactone[15], sorgolactone[8] and strigol.[9] Based on CD spectra the absolute configuration at C2'of **(+)-7** is *R* and of **(-)-7** is *S*. This assignment is in accordance with the germination stimulatory activity towards seeds of *Striga hermonthica*. The diastereoisomer **(+)-7** having the natural configuration at all its stereogenic centres shows an appreciable activity at a concentration of 10^{-8}M, whereas its antipode **(-)-7** hardly exhibits any activity at the same concentration (see table 1). In addition, an X-ray analysis

was performed on the slow moving diastereoisomer derived from tricyclic lactone **(-)-5**. A PLUTON generated drawing of the crystal structure of **(+)-8** is shown in Fig. 3. It should be noted that the refinement process of this X-ray analysis needed special care to obtain consistent values for the average displacement parameters, distances and bond angles. This diastereoisomer **(+)-8** has *S* configuration at C2', which stereochemistry at the B-C ring junction is the same as in **(+)-7**.

Figure 3

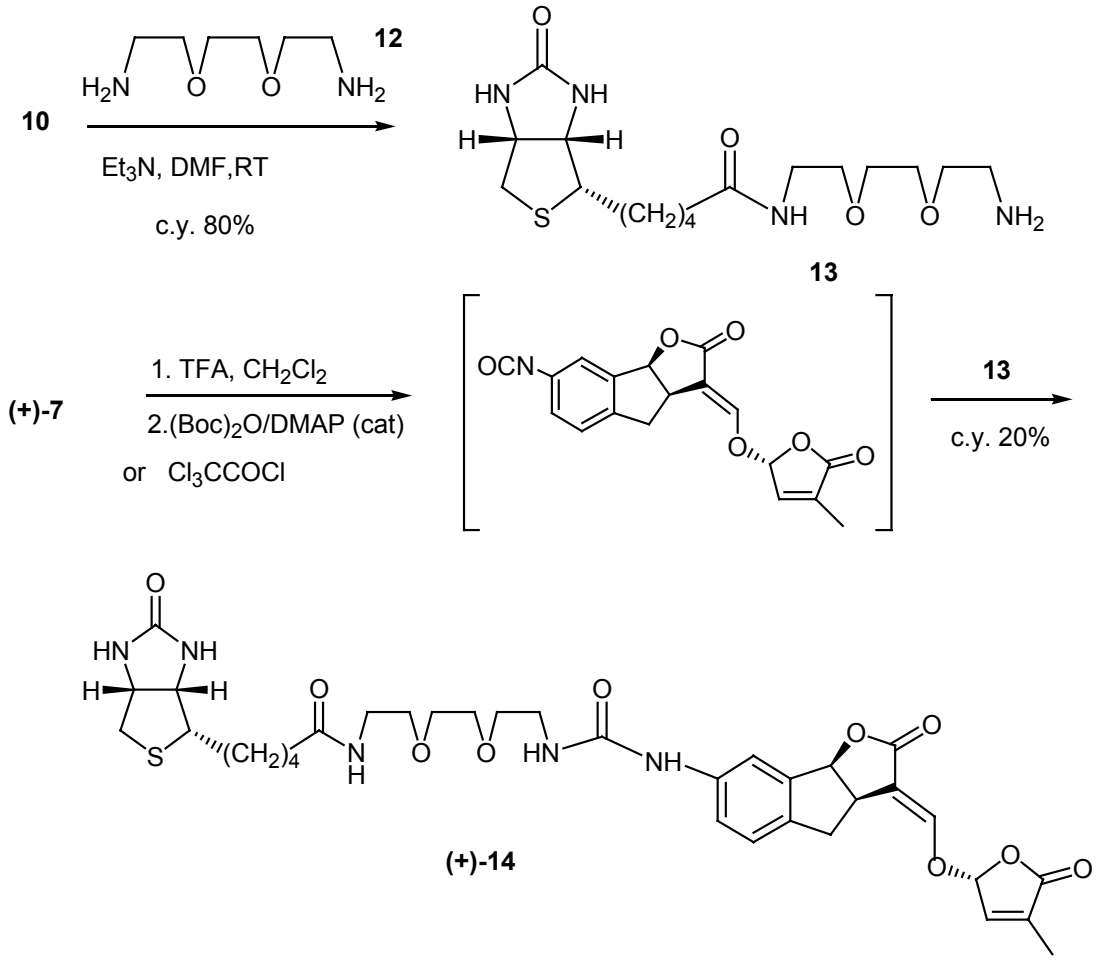


Scheme 2



The coupling of diastereoisomer *rac*-**7** with biotin was investigated next. Removal of the Boc group, followed by coupling with biotin **9** using DCC/DMAP did not meet with success. Therefore, an activated ester, namely the pentafluorophenyl (Pfp) ester[31] i.e. of biotin **9**, **10,** was considered. Gratifyingly, a smooth coupling with amino-GR24 took place in the presence of HOBT, to give the product **11** as an inseparable mixture of diastereoisomers in 72% yield (Scheme 2).

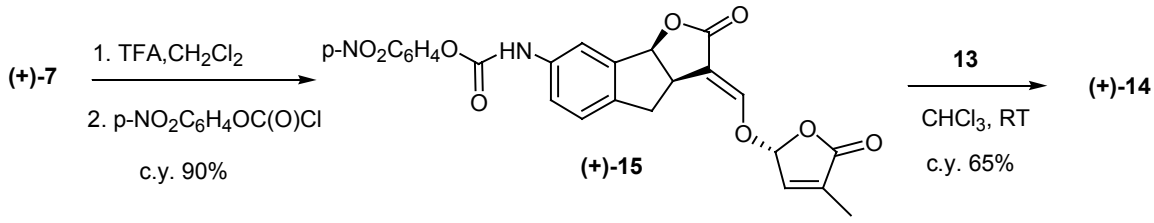
Scheme 3



Then attempts were made to incorporate a linking spacer **12**. This linker **12** was chosen in order to improve the water solubility of the biotin derivative. The coupling of biotin Pfp ester **10** with bisamine **12** gave amide **13** following a slightly modified literature procedure.[32] Coupling of **13** with amino-GR24 was accomplished via the isocyanate derived from amino-GR24 upon reaction with $(Boc)_2O$ or trichloroacetyl chloride. Starting

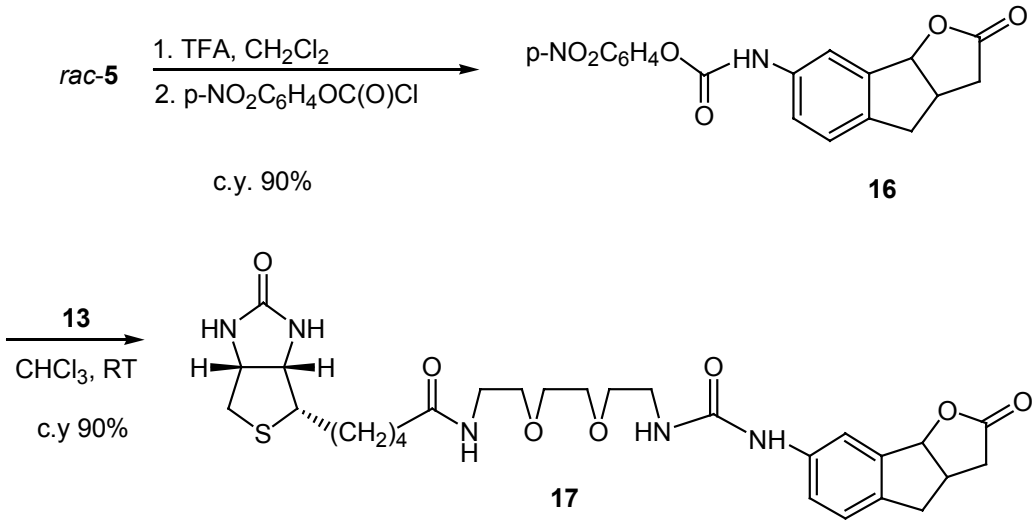
from diastereoisomer (+)-**7** biotin labelled compound (+)-**14** was obtained in a modest yield of 20% (Scheme 3). Conversion of the biotin substrate **13** into the corresponding isocyanate and its subsequent *in sito* coupling with (+)-**7** gave unsatisfactory results. However, the alternative coupling of p-nitrophenyl carbamate (+)-**15** obtained from **(+)-7** by reaction with p-nitrophenyl chloroformate with **13** resulted in the desired biotin labelled GR24 analogue in a good yield (65%) (Scheme 4).

Scheme 4



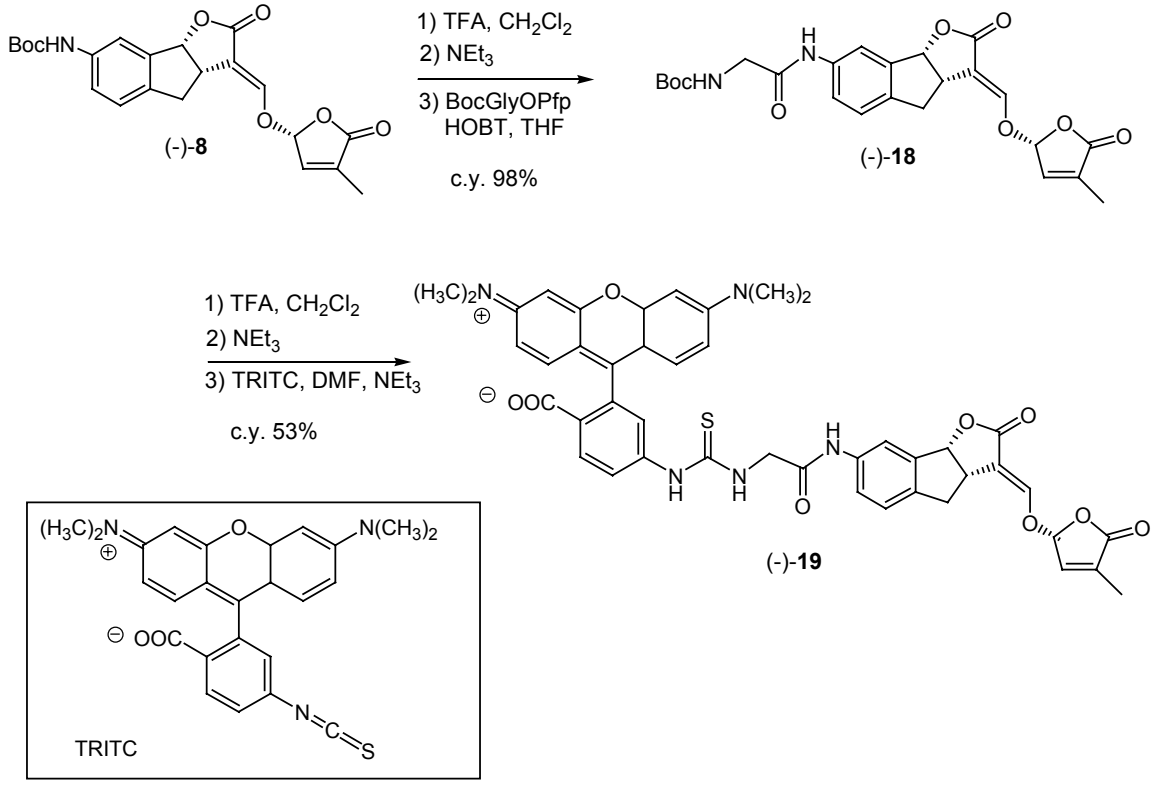
In order to exclude non-specific binding during the receptor protein identification procedure, a negative control must be included. Biotin labeled ABC-lactone **17** is a suitable candidate for this purpose as it is lacking the vinyl enol-ether moiety and the D-ring, which are proposed as the bioactiphore for the strigolactone receptor[14] (Scheme 5). Compound **17** was prepared in the same manner as described for (+)-**14** again using a p-nitrophenyl carbamate viz. **16.** *Rac*-**5** gave an inseparable mixture of diastereoisomers of **17** in 90% yield (Scheme 5).

Scheme 5



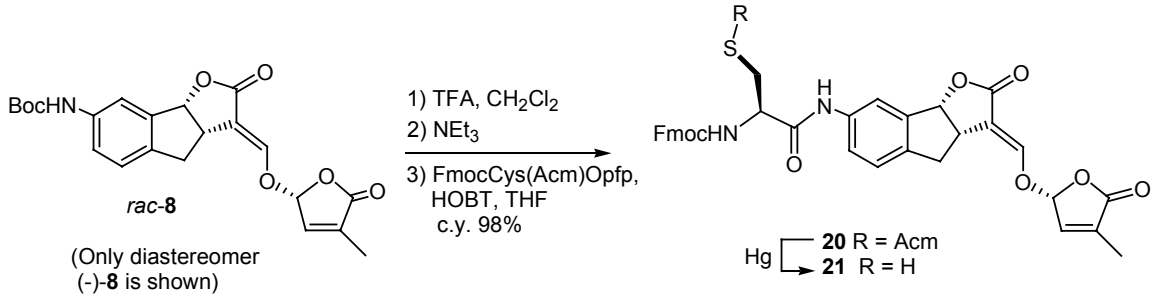
A labelled strigolactone analogue suitable for FCS studies requires a fluorescent tag with an absorption maximum at the wavelength of the FCS-laser. Tetramethyl rhodamine (λ_{max}Abs = 543 nm) is suited for excitation at the 543 nm spectral line of the green Helium-Neon laser.[33] The reactive isothiocyanate of tetramethyl rhodamine (TRIC) was chosen for coupling to amino-GR24 derived from **(-)-8**.[34b] However, the aromatic amine group was not very reactive towards TRIC. Therefore, a glycine spacer was linked to the aromatic amine, which was readily performed via coupling with the PfP ester of glycine in the presence of HOBT (Scheme 6). Removal of the Boc protective group from **(-)-18**, gave a highly reactive primary amine, which smoothly reacted with TRIC. Isolation of the intermediate primary amine was not possible, because of its rapid conversion to the corresponding carbamic acid by reaction with CO_2 in air. Fluorescently labelled strigolactone analogue **(-)-19** was purified by silicagel chromatography and isolated as a bright pink powder in 53% yield.[34b]

Scheme 6



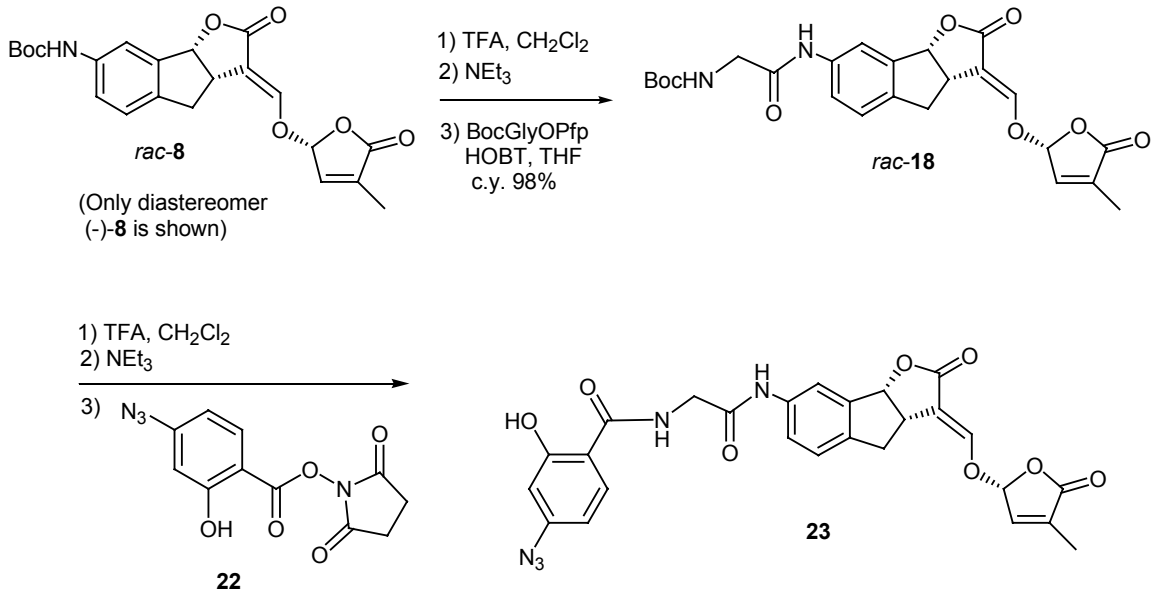
A ligand that can be used as a probe in SFM, requires the presence of an SH group. Since coupling of the amine derived from *rac*-**8** with an amino acid PfP ester proceeded smoothly, this reaction was also chosen for the introduction of the SH moiety into the GR24 derivative. Commercially available cysteine derivative FmocCys(Acm)OPfP was reacted *in situ* with the aromatic amine to give compound **20** (Scheme 7).[34b] The acetamido methyl (Acm) protective group can be removed under natural conditions in the presence of mercury[35]. In principle, ligand **21** can be linked to a gold SFM tip. The position of the SH group in the ligand is such that steric interference with the bioactiphore of the strigolactone analogue is unlikely. It is not strictly necessary to remove the Fmoc group, provided the molecule is sufficiently active in the germination of parasitic weed seeds. The synthetic scheme for **21** can also be used for enantiomerically pure material, which then can be employed to coat the gold probe for the SFM-study.

Scheme 7



PAL needs a ligand containing a photoreactive group. It is highly desirable to include this group in the final synthetic step, to minimize the risk of premature activation of the light sensitive unit. To demonstrate the feasibility of the synthetic strategy, succinimidyl ester **22** was reacted with strigolactone analogue *rac*-**18** after deprotection (Scheme 8).[34] The coupling with the aliphatic primary amine of the spacer proceeded in quantitative yield, whereas the aromatic amino-GR24 was much less reactive.[34]

Scheme 8



Biological activity

The germination stimulatory activity of strigolactones (+)-**7**, (-)-**7**, (+)-**14, 17**, (-)-**19**[34b] and **20**[34b] was assayed using seeds of *Striga hermonthica*. In all germination assays, an aqueous solution of acetone (0.1% v/v) was included as a negative control and a diastereomeric 1:1 mixture of GR 24 as a positive control. This procedure enables comparison between results obtained in different test series. The results for *S. hermonthica* are collected in table 1. PAL-ligand **23** was not included in these tests. It was not possible to determine the biological activity due to its insolubility in an aqueous medium, even when a large amount of cosolvent was used to prepare the stock solution of this compound.[34] For *S. hermonthica*, labelled compounds (+)-**14**, **19** and **20** stimulated the germination of the weed seeds, confirming that a large A-ring substituent is indeed tolerated by the strigolactone binding site of *S. hermonthica* seeds. As expected compound **17** exhibit no activity in these seeds. It should be noted however, that compound **20** was also rather insoluble in aqueous medium. The germination values reported for this compound were difficult to reproduce, probably due to crystalisation of the stimulant in the aqueous solution. [34b] In all assays, the aqueous blanks did not induced germination of the seeds.

Fluorescent stimulant (-)-**19** dissolved well in water because of its ionic nature. [34]

Interestingly, compounds **(-)-19** and **20** which were prepared from the diastereoisomer **(-)-8**,[34] were found to induce germination of the seeds of *Striga hermonthica*. Diastereoisomer **(-)-8** possesses the same stereochemistry as the natural strigol at C'2, but opposite stereochemistry at C8b and C3a. However, it was demonstrated earlier that the stereochemistry at C2' is essential for biological activity, whereas the stereochemistry at C8b and C3a has a minor effect on the germination.[15] Furthermore, the lack of a significant reduction in the stimulation activity found with these compounds (see table 1) suggests that they would be suitable candidates for isolation of the strigolactone receptor.

In summary, labelled strigolactone analogues tagged with a fluorescent, radioactive, photoaffinity moiety and biotin group suitable for the isolation and identification of the strigolactone receptor, were prepared. Amino-GR24 is the appropriate starting material for the attachment of various tags to the A-ring. Bioassays of the labelled stimulants reveal that the germination activity on seeds of *Striga hermonthica* is mostly retained. Preliminary results in the isolation of strigolactone receptor, using the synthetic biotinylated strigolactone analogues, showed a binding protein present in the membrane fractions of *Striga hermonthica* seeds. These results will be reported elsewhere in due time[36].

*Table 1 Percentages of germinated seeds of S. hermonthica after exposure to solutions (2×10^{-6} and 2×10^{-8} mol/L) of (+)-**7**, (-)-7, (+)-**14**, **17**, (-)-**19**, **20** relative to the control GR24 (**3**).*[a]

entry	compound	% germination ± SE at a concentration of 2×10^{-6}	2×10^{-8} mol/L
1	**(+)-7**	78±5.1	90±6.2
2	**(-)-7**	68±4.5	10±1.2
3	**(+)-14**	61±2	70±5
4	**17**	4 ± 1.1[c]	3 ± 1.1[c]
5	(-)-**19**	130±4.9	66±0.7
6	**20**	74±8.1	37±3.6
7[d]	**GR24**[b]	100	100

[a]Data presented the mean ± SE of one representative experiment and are relative to the GR24. [b]Equimolar mixture of two racemic diastereomers. [c] Not significantly different from aqueous control (without stimulant).[d] The average germination of GR24 was found to be in a range of 50-60% .

5.3 Experimental

Enantiomeric excesses of compounds **7** and **8** were determined by analytical HPLC using Chiralcel OD (10 μm) cellulose carbamate column (Baker, 250 × 4.6 mm) using mixtures of 2-propanol or ethanol and hexane as the eluent. CD-spectra were recorded using a Jasco spectrophotometer.

Synthesis

7-Nitro-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-2-one

Tricyclic lactone **(-)-4** (2.9 g, 16.7 mmol) was nitrated as described by Thuring *et al.* [20] Recrystallization from toluene afforded pale yellow needles. Yield: 74%. Mp: 118-119°C. $[\alpha]^{22}_{D}$ -197° (c=0.4, CH_2Cl_2). All other analytical data were identical with those reported previously for the racemic compound.[20]

7-Nitro-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-2-one

Tricyclic lactone **(+)-4** (3.25 g, 18.7 mmol) was nitrated as described by Thuring *et al*[20] Recrystallization from toluene afforded pale yellow needles. Yield: 75%. Mp: 119-120°C. $[\alpha]^{22}_{D}$ +207.8° (c=0.4, CH_2Cl_2). All other analytical data were identical with those reported previously for the racemic compound.[20]

***tert*-Butyl *N*-[2-oxo-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (-)-5**

Reduction of nitro tricyclic lactone **(-)-4** (2.66 g, 12.15 mmol) was accomplished in quantitative yield with tin and hydrochloric acid as described previously for the racemic compound[20]. A mixture of 7-Amino-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-2-one (2.0 g, 10.6 mmol) and di*tert*-butyl dicarbonate (3.5 g, 16 mmol) was heated under reflux in THF (25 mL). After 2.5 h, the mixture was cooled and THF was removed under reduced pressure. The residue was dissolved in ethyl acetate, washed with tartaric acid (1M, 1×), dried ($MgSO_4$) and concentrated. Silicagel chromatography (hexane/ethyl acetate

1/1) yielded **(-)-5** (2.6 g, 85%) as a white solid. Pure **(-)-5** was obtained by crystallization from toluene following by second recrystallization from hexane/ethyl acetate mixture afforded colourless needles. Mp: 135-136°C. $[\alpha]^{22}_{D}$ -158.5° (c=0.4, CH_2Cl_2). ^{1}H NMR (300MHz, $CDCl_3$): δ 1.52 (s, 9H, 3 × C*H*$_3$ tBu), 2.37 (dd, 1H, J_1 = 5.8Hz J_2 = 18.0Hz, *H*3), 2.82 (dd, 1H, J_1 = 3.4Hz, J_2 =16.3Hz, *H*4), 2.88 (dd, 1H, J_1 = 9.8Hz, J_2 = 18.0Hz, *H*3), 3.25 (dd, 1H, J_1 = 8.3Hz, J_2 = 16.3Hz, *H*4), 3.37 (m, 1H, *H*3a); 5.83 (d, 1H, *J* = 7.2Hz, *H*8b), 6.54 (bs, 1H, N*H*); 7.17 (d, 1H, *J* = 8.2Hz, *H*5), 7.33 (d, 1H, *J* = 8.3Hz, *H*6), 7.51 (s, 1H, *H*8). ^{13}C NMR ($CDCl_3$): δ 28.2 (3C, *C*H$_3$ tBu), 35.6, 37.3 (2C, *C*H$_2$); 37.7 (1C, *C*H3a), 80.7 (1C, *C*q tBu), 87.5 (1C, *C*H8b), 116.3, 120.8, 125.6 (3C, *C*H$_{arom}$), 136.8, 138.0, 139.6, (3C, *Cq*$_{arom}$), 141.7 (1C, *C*H3'), 152.7, 176.8 (2C, CO). IR (KBr): ν (cm^{-1}) 3338, 1756, 1726,1180. MS [EI *m/z*, rel. intensity (%)]: 289 ([M]$^+$, 23.8), 233([$C_{12}H_{11}O_4N$]$^+$, 14.5), 189([$C_{11}H_{11}O_2N$]$^+$, 24.0), 57([C_4H_9]$^+$, 81.3), 28([CO]$^+$, 100). Anal. calcd. for $C_{16}H_{19}O_4N$: C, 66.42; H, 6.62; N, 4.84 found: C, 66.20; H, 6.60; N, 4.87.

***tert*-Butyl *N*-[oxo-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (+)-5**

Boc-amino tricyclic lactone **(+)-5** was prepared in the same way as **(-)-5**, starting from 7-nitro-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-2-one **(+)-4** (3.0 g, 15.9 mmol). An analytically pure sample was obtained by recrystallilization from hexane/ethyl acetate. Yield 87%. $[\alpha]^{22}_{D}$ +160.2° (c=0.4, CH_2Cl_2). Anal. calcd. for $C_{16}H_{19}O_4N$: C, 66.42; H, 6.62; N, 4.84 found: C, 66.23; H, 6.57; N, 4.88. All other analytical data were the same as reported for carbamate (-)-**5**.

***tert*-Butyl *N*-[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*RS*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*RS*),4,8b(*SR*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (*rac*-7)** and its 2(S) diastereoisomer: ***tert*-Butyl *N*-[3-((*E*)-1-{[4'-methyl-5'oxo-2',5'-dihydro-2'(*SR*)-furanyl]oxy}-methylidene)-2-oxo-3,3a(*RS*), 4,8b(*SR*) -tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (*rac*- 8)**

Racemic Boc-amino-GR24 diastereoisomers *rac*-**7** and *rac*-**8** were prepared in the same way as described for optically pure compounds (-)-**7** and (-)-**8**, (*vide infra*) starting from carbamate *rac*-**5** (1.0 g, 3.46 mmol). Yield: fast moving diastereoisomer *rac*-**7**: 39%; slow moving diastereoisomer *rac*-**8**: 37%. All analytical data were the same as reported for the corresponding enantiopure carbamates.

***tert*-Butyl *N*-[3-((*E*)-1-{[4'-methyl-5'oxo-2',5'-dihydro-2'(*R*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate ((-)-7** and its 2(*S*) diastereoisomer: ***tert*-Butyl *N*-[3-((*E*)-1-{[4'-methyl-5'oxo-2',5'-dihydro-2'(*S*)-furanyl]oxy}-methylidene)-2-oxo-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (-)-8**

To a cooled (0°C) and stirred solution of Boc-amino tricyclic lactone (+)-**5** (650 mg, 2.25 mmol) in ethyl formate (10 ml) was added, under continuous stream of nitrogen, 2.2 equiv. of metallic sodium (111 mg, 4.8 mmol). The mixture was allowed to warm to room temperature and stirred for 1.5 hour. When TLC analysis indicated complete formylation excess ethyl formate was removed by evaporation *in vacuo*. The thus obtained sodium salt of formylated **(+)-5** was suspended in THF (10 mL) and cooled to 0^{o}C. Upon addition of chlorobutenolide **6** (583 mg, 4.4 mmol) the reaction mixture became clear. The mixture was stirred overnight. Then THF was removed *in vacuo* and the residue was dissolved in a mixture of brine and ethyl acetate. The aqueous phase was extracted with ethyl acetate (2 × 20 mL) and combined organic layers were washed with saturated NH_4Cl (20 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified using flash chromatography (SiO_2, hexane/ethyl acetate 2/1) to afford two diastereoisomeric products. Fast moving diastereoisomer (-)-**(7)** (400 mg, 44%) and slow moving diastereoisomer (-)-**8** (380 mg, 42%) were obtained as white solids after recrystallization from hexane/ethyl acetate.

(-)-7: mp: 115-117°C. Ee >99% (determined by HPLC). $[\alpha]^{22}_{D}$= -331.6° (c=0.1, CH_2Cl_2). ^{1}H NMR (300MHz, $CDCl_3$): δ 1.51 (s, 9H, 3 × C*H*$_3$ tBu); 2.04 (s, 3H, C*H*$_3$ D-ring), 3.04 (dd, 1H, J_1= 3.2 Hz, J_2 = 16.7 Hz, *H*4), 3.37 (dd, 1H, J_1 = 9.3Hz, J_2 = 16.7Hz, *H*4), 3.94 (m, 1H, *H*3a), 5.90 (d, 1H, *J* = 7.9Hz, *H*8b), 6.17 (m, 1H, *H*2'), 6.50 (bs, 1H, N*H*), 6.96 (m,

1H, *H*3'), 7.15 (d, 1H, *J* = 8.3Hz, *H*5), 7.35 (dd, 1H, J_1 = 8.3Hz, J_2 = 1.7Hz, *H*6), 7.47 (d, 1H, *J* = 2.5Hz, *H*6'), 7.50 (d, 1H, *J* = 1.7Hz, *H*8). ^{13}C NMR ($CDCl_3$): δ 11.3 (*C*H_3 D-ring), 28.9 (3C, *C*H_3 tBu), 37.3 (1C, *C*H_2), 39.9 (1C, *C*H3a), 81.1 (1C, *Cq* tBu), 86.4 (1C, *C*H8b), 101.3 (1C, *C*H2'), 113.7 (1C, *Cq* C-ring), 116.9, 121.6, 126.0 (3C, *C*H_{arom}); 136.3, 137.5, 138.6, 140.2 (3C, Cq_{arom} and 1C, Cq D-ring), 141.7 (1C, CH3'), 151.7 (1C, CH6'), 153.4, 170.9, 171.8 (3C, CO). MS [CI *m/z* rel. intensity (%)]: 413 ($[M]^+$, 12.5); 357 ($[C_{18}H_{15}O_7N]^+$,88.8); 313 ($[C_{17}H_{15}O_5N]^+$, 9.3); 217 ($[C_{12}H_{11}O_3N]^+$, 21.4); 97 ($[C_5H_5O_2]^+$, 32.2), 57 ($[C_4H_9]^+$,100). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 64.06; H, 5.73; N, 3.31.

(-)-8: mp: 226-228°C (decomp.). Ee >99% (determined by HPLC). $[\alpha]^{22}_D$= -323.6° (c=0.1, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.53 (s, 9H, 3 × C*H*$_3$ tBu), 2.04 (s, 3H, C*H*$_3$ D-ring), 3.03 (dd, 1H, J_1 = 3.1Hz, J_2 = 16.7Hz, H4), 3.35 (dd,1H, J_1 = 9.2Hz, J_2 = 16.7Hz, *H*4), 3.93 (m, 1H, *H*3a), 5.90 (d, 1H, *J* = 7.8Hz, *H*8b), 6.16 (m, 1H, *H*2'); 6.48 (bs, 1H, N*H*), 6.96 (m, 1H, *H*3'), 7.13 (d, 1H, *J* = 8.3Hz, *H*5), 7.30 (dd, 1H, J_1 = 8.3Hz, J_2 = 1.8Hz, *H*6), 7.46 (d, 1H, *J* = 2.5Hz, *H*6'), 7.53 (d, 1H, *J* = 1.8Hz, *H*8). ^{13}C NMR ($CDCl_3$): δ 11.4 (1C, *C*H_3 D-ring), 29.0 (3C, *C*H_3 tBu), 37.5 (1C, *C*H_2), 39.9 (1C, *C*H3a), 81.3 (1C, *Cq* tBu), 86.5 (1C, *C*H8b), 101.3 (1C, *C*H2'), 114.0 (1C, *Cq* C-ring), 116.9, 121.7, 126.2 (3C, *C*H_{arom}), 136.6, 138.5, 138.6, 140.3 (3C, *Cq*$_{arom}$ and *Cq* D-ring), 141.7 (1C, *C*H3'), 151.6 (1C, *C*H6'), 153.4, 170.9, 171.9 (3C, *C*O). MS [CI *m/z* rel. intensity (%)]: 413 ($[M]^+$,15.8); 357 ($[C_{18}H_{15}O_7N]^+$,44.7); 313 ($[C_{17}H_{15}O_5N]^+$,25.0); 217 ($[C_{12}H_{11}O_3N]^+$, 73.4); 97 ($[C_5H_5O_2]^+$, 68.2), 57 ($[C_4H_9]^+$,100). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 64.06; H, 5.70; N, 3.21.

tert-Butyl _N_-[3-((_E_)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(_S_) furanyl]oxy} methylidene)-2-oxo-3,3a(_S_),4,8b(_R_)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (+)-7 and its 2(*R)* diastereoisomer: **_tert_-Butyl _N_-[3-((_E_)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(_R_)-furanyl]oxy}-methylidene)-2-oxo-3,3a(_S_),4,8b(_R_)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (+)-8**

Boc-amino-GR24 diastereoisomers **(+)-7** and **(+)-8** were prepared in the same way as described for their enantiomers **(-)-7** and **(-)-8,** starting from carbamate **(-)-5** (770 mg, 2.7

mmol)**.** Yield **(+)-7:** 43%; **(+)-8:** 38%. Analytical samples were obtained by recrystallization from 2-methyl-2-butanol.

(+)-7: mp: 116-118°C. Ee >99% (determined by HPLC). $[\alpha]^{22}_{D}$=+338.7° (c=0.1, CH_2Cl_2). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 64.10; H, 5.73; N, 3.13. All other analytical data were the same as reported for **(-)-7**.

(+)-8: mp: 228-230°C. Ee >99% (determined by HPLC). $[\alpha]^{22}_{D}$= +331.5° (c=0.1, CH_2Cl_2). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 63.63; H, 5.62; N, 3.44. All other analytical data were the same as reported for **(-)-8**.

_N_1-[(3a_R_,8b_S_)-3-((_E_)-1-[(2_R_)-4-methyl-5-oxo-2,5-dihydro-2-furanyl]oxymethylidene)-2-oxo-3,3a,4,8b-tetrahydro-2_H_-indeno[1,2-_b_]furan-7-yl]-5-(2-oxoperhydrothieno[3,4-_d_]imidazol-4-yl)pentanamide 11

Complete deprotection of *rac*-**7** (20 mg, 0.064 mmol), obtained in the same manner as **(-)-7**, starting from *rac*-**5**, using 1.5 mL TFA and 10 mL CH_2Cl_2 after 1 h. After evaporation to remove the excess acid, EtOAc was added and pH 8 was adjusted by addition of saturated $NaHCO_3$. The organic layer was then washed with little cold water and dried ($MgSO_4$). Volatiles were evaporated and the free amine was dissolved in dry acetonitrile (20 mL). Then **10**[31] (28 mg, 0.068 mmol) and hydroxy benzotriazole (HOBT, 10 mg, 0.07 mmol) were added and the mixture was stirred at room temperature for 12 h. Acetonitrile was removed *in vacuo* and the residue was purified by column chromatography (SiO_2; CH_3OH/CH_2Cl_2 7:1) to give **11** as a white solid (20 mg, 72% yield). TLC: ($CHCl_3$/MeOH 5:1, R_f = 0.31). ^{1}H NMR (300MHz, $CDCl_3$): δ 1.60 (m, 6H, biotin-CH_2), 2.03 (s, 3H, CH_3 D-ring), 2.33 (t, 2H, J = 7.5Hz, CH_2CO), 2.70 (d, 1H, J = 12.6Hz, CHH_{endo}S), 2.84 (dd, 1H, J_1 = 12.6 Hz, J_2 = 4.5Hz, 1H, CHH_{exo}S), 3.08 (m, 2H, CHS and H4), 3.33 (dd, 1H, J_1 = 16.7Hz, J_2 = 9.3Hz, H4), 3.91 (m, 1H, H3a), 4.31, 4.49 (m, 2H, CHNH), 5.32, 5.51

(2s, 1H, N*H*), 5.88 (d, J_1 = 7.5 Hz, 1H, *H*8b), 5.97 (brt, 1H, N*H*), 6.21 (s, 1H, *H*2*'*),6.33, 6.79 (s, 2H, N*H*), 6.99 (m, 1H, *H*3'); 7.11 (d, 1H, *J* = 8.4 Hz, *H*5), 7.48 (m, 1H, *H*8), 7.59 (d, 1H, *J* = 9.0 Hz, *H*6), 7.69 (m, 1H, *H*6), 8.86 (s, 1H, N*H*). ^{13}C NMR ($CDCl_3$): δ 174.18, 171.8, 170.9, 163.97, 156.25 (5C, *C*O), 151.58 (1C, *C*H6'), 141.1 (1C, *C*H3'), 139.64, 139.28, 135.77, 135.69 (4C, 3Cq_{arom} and Cq D-ring), 125.37, 121.38, 116.16 (3C, *C*H_{arom}), 112.96 (1C, *Cq* C-ring), 100.73 (1C, *C*H2'), 86.15 (1C, *C*H8b), 61.79, 60.13 (2C, *C*HNH), 55.41 (1C, *C*HS), 40.44 (1C, *C*H_2S), 39.72 (1C, *C*H3a), 39.41, 39.29 (2C, *C*H_2NH), 36.55 (1C, *C*H_2), 35.98 (1C, *C*H_2CO), 28.05, 27.91, 25.48 (3C, *C*H_2), 22.85 (1C, *C*H_3 D-ring); MS [FAB *m/z*, rel. intensity (%)]), 539 ([M]$^+$, 100). Anal. calcd. for $C_{27}H_{29}N_3O_7S$: C, 60.10; H, 5.42; N, 7.79 found: C, 60.36; H, 5.73; N, 7.90.

N1-2-[2-(2-aminoethoxy)ethoxy]ethyl-5-(2-oxoperhydrothieno[3,4-*d*]imidazol-4-yl)pentanamide 13

A solution of **10**[31] **(**0.5 g, 1.21 mmol) in anhydrous DMF (25 mL) was added dropwise over 1 hour to a mixture of aminoethoxyethoxyethylamine **12** (2.0 mL, 12 mmol), and Et_3N (0.5 mL, 3.6 mmol). The reaction was stirred at room temperature for 30 min and the solution was concentrated *in vacuo*. The resulting oil was triturated in 50 mL ether and filtered. The crude mixture was purified by a counter current extraction (Butanol:water 1:1) and freeze-dried. TLC control (Butanol/water/acetic acid 4:1:1) showed almost no bis-substituted diamine. The thus obtained white solid (0.4 g, 87% yield with respect to biotin) was used without further purification. TLC (Butanol/water/acetic acid 4:1:1 R_f = 0.25). ^{1}H NMR (300MHz, DMSO-d_6): δ 1.4 (m, 6H, biotin-CH_2), 1.6 (s, 2H, NH_2), 2.06 (t, 2H, *J* = 7.5Hz, CH_2CO), 2.52 (d, 1H, *J* = 12.45Hz, CHH_{endo}S), 2.64 (t, 2H, *J* = 6 Hz, CH_2-NH_2), 2.81 (dd, 1H, J_1 = 12.0 Hz, J_2 = 4.8Hz, 1H, CHH_{exo}S), 3.10 (m, 1H, C*H*S), 3.16 (m, 2H, CH_2NH), 3.3 (m, 4H, OCH_2), 3.5 (s, 4H, OCH_2CH_2O), 4.29, 4.11 (m, 2H, C*H*NH), 6.42, 6.35 (s, 2H, N*H*), 7.85 (t, 1H, *J* = 5.7 Hz N*H*CO). ^{13}C NMR (DMSO-d_6): δ 172.12, 162.70 (2C, *C*O), 72.80, 69.55, 69.51, 69.15 (4C, *C*H_2O), 61.02, 59.17 (2C, *C*HNH), 55.41 (1C, *C*HS), 41.21 (1C, *C*H_2S), 38.44 (2C, *C*H_2NH), 35.08 (1C, *C*H_2CO), 28.18, 28.03, 25.26 (3C, *C*H_2); IR (KBr): ν (cm^{-1}) 3289, 3081, 2927, 2862, 1701, 1645, 1553, 1461, 1423, 1262, 1119 .- MS (FAB/NBA): *m/z* = 375 [M^+] (100%).

_N_1-2-[2-(2-[(3a_R_,8b_S_)-3-((_E_)-1-[(2_S_)-4-methyl-5-oxo-2,5-dihydro-2-furanyl]oxymethylidene)-2-oxo-3,3a,4,8b-tetrahydro-2_H_-indeno[1,2-_b_]furan-7-yl]aminocarbonylamino]ethoxy)ethoxy]ethyl-5-(2-oxoperhydrothieno[3,4-_d_]imidazol-4-yl)pentanamide. (+)-**14**

Complete deprotection of **(**+**)-7** (46 mg, 0.11 mmol) was achieved with 1.5 mL TFA and 10 mL CH_2Cl_2 after 1 h. After evaporation to remove the excess acid, EtOAc was added and pH 8 was adjusted by addition of saturated $NaHCO_3$. The organic layer was then washed with little cold water and dried ($MgSO_4$). Volatiles were evaporated and the free amine was dissolved in dry CH_2Cl_2 (20 mL). Then 1.05 equiv. of p-nitorophenyl chloroformate (23 mg, 0.115 mmol) and 1.05 equiv. of pyridine (10 μL, 0.115 mmol) were added and the mixture was stirred at room temperature for 12 h. The reaction mixture was diluted with $CHCl_3$ (100 mL), washed with 1% $KHSO_4$ and dried ($MgSO_4$) to obtain (42 mg, 80%) of yellow solid after recrystallization from ethyl acetate/hexane. To a solution of crude carbamate (17 mg, 0.048 mmol) in 2 mL $CHCl_3$, **13** (13 mg, 0.048 mmol) and Et_3N (6.7 μL, 0.048 mmol) were added. TLC control ($CHCl_3$/MeOH 5:1) after 15 min showed complete conversion. Solvent was removed *in vacuo*. Purification by column chromatography (SiO_2, $CHCl_3$/MeOH 7:1) gave 23 mg of product (+)-**14** as a white solid (89% yield) after precipitation from diethyl ether. TLC: ($CHCl_3$/MeOH 5:1, R_f = 0.44). ^{1}H NMR (300MHz, $CDCl_3$): δ 1.45 (m, 6H, biotin-C*H*$_2$), 2.03 (s, 3H, C*H*$_3$-Dring), 2.06 (t, 2H, *J* = 7.5Hz, C*H*$_2$CO), 2.70 (d, 1H, *J* = 12.6Hz, CH*H*$_{endo}$S), 2.84 (dd, 1H, J_1 = 12.6 Hz, J_2 = 4.5Hz, 1H, CH*H*$_{exo}$S), 3.06 (m, 2H, C*H*S and H4), 3.33 (dd, 1H, J_1 = 16.8Hz, J_2 = 9.3Hz, H4) 3.44 (m, 4H, OC*H*$_2$), 3.55 (m, 2H, C*H*$_2$NH), 3.62 (s, 4H, OC*H*$_2$C*H*$_2$O), 3.91 (m, 1H, *H*3a), 4.23, 4.46 (m, 2H, C*H*NH), 5.29 (s, 1H, N*H*), 5.88 (d, J_1 = 7.8 Hz, 1H, *H*8b), 5.97 (brt, 1H, N*H*), 6.21 (s, 1H, *H*2*'*), 6.33, 6.79 (s, 2H, N*H*), 6.99 (m, 1H, *H*3'), 7.11 (d, 1H, *J* = 9.0 Hz, *H*5), 7.40 (m, 1H, *H*8), 7.52 (d, 1H, *J* = 2.4 Hz, *H*6'), 7.60 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz, *H*6), 8.43 (s, 1H, N*H*). ^{13}C NMR ($CDCl_3$): δ 174.18, 171.8, 170.9, 163.97, 156.25 (5C, *C*O), 151.58 (1C, *C*H6'), 141.1 (1C, *C*H3'), 139.64, 139.28, 135.77, 135.69 (4C, 3*Cq*$_{arom}$, Cq D-ring), 125.37, 121.38, 116.16 (3C, *C*H$_{arom}$), 112.96 (1C, *Cq* C-ring), 100.73 (1C, *C*H2'), 86.15 (1C, *C*H8b), 70.71, 70.52, 69.75, 69.68 (4C, *C*H$_2$O), 61.79, 60.13 (2C, *C*HNH), 55.41 (1C, *C*HS), 40.44 (1C, *C*H$_2$S), 39.72 (1C, *C*H3a), 39.41, 39.29

(2C, *C*H_2NH), 36.55 (1C, *C*H_2), 35.98 (1C, *C*H_2CO), 28.05, 27.91, 25.48 (3C, *C*H_2), 22.85 (1C, *C*H_3 D-ring); Anal. calcd. for $C_{34}H_{43}N_5O_{10}S$: C, 57,21; H, 6,07; N, 9,81 found: C, 60.36; H, 5.73; N, 7.90.

_N_1-[2-(2-2-[(2-oxo-3,3a,4,8b-tetrahydro-2_H_-indeno[1,2-_b_]furan-7-yl)aminocarbonylamino]ethoxyethoxy)ethyl]-5-(2-oxoperhydrothieno[3,4-_d_]imidazol-4-yl)pentanamide 17

Compound **17** was prepared in the same way as (+)**-14**, starting from *rac*-**5** (100 mg, 0.34 mmol). Purification by column chromatography (SiO_2, $CHCl_3$/MeOH 7:1) gave 23 mg of product **14** as a white solid (89% yield calculated on the carbamate) after precipitation from diethyl ether. TLC: ($CHCl_3$/MeOH 5:1, R_f = 0.44). ^{1}H NMR (400MHz, $CDCl_3$): δ 1.45 (m, 6H, biotin-C*H*$_2$), 2.21 (t, 2H, *J* = 7.5Hz, C*H*$_2$CO), 2.37 (ddd, J_1 = 20 Hz, J_2 = 4.9 Hz, J_3 = 2.4Hz, *H*3), 2.72 (dd, 1H, J_1 = 12.6Hz, J_2 = 3.2 Hz, CHH_{endo}S), 2.84 (dd, 1H, J_1 = 12.6 Hz, J_2 = 4.5Hz, 1H, CHH_{exo}S), 2.88 (dd, J_1 = 18 Hz, J_2 = 9.8 Hz, *H*3), 3.09 (m, 2H, C*H*S and *H*4), 3.21 (dd, 1H, J_1 = 16.4Hz, J_2 = 8.5 Hz, *H*4), 3.33 (m, 1H, *H*3a), 3.44 (m, 4H, OC*H*$_2$), 3.56 (m, 2H, C*H*$_2$NH), 3.62 (s, 4H, OC*H*$_2$C*H*$_2$O), 4.26, 4.48 (m, 2H, C*H*NH), 5.55, 5.57 (2s, 1H, N*H*), 5.81 (d, J_1 = 7.5 Hz, 1H, *H*8b), 6.01 (t, *J* = 4.9 Hz, 1H, N*H*), 6.59, 6.69 (s, 2H, N*H*), 6.94 (m, 1H), 7.13 (d, 1H, *J* = 8.3 Hz, H5), 7.40, 7.42 (2m, 1H, *H*8), 7.60 (dd, 1H, J_1 = 9.3 Hz, J_2 = 1.9 Hz, *H*6), 8.37 (s, 1H, N*H*). ^{13}C NMR ($CDCl_3$): δ 177.32, 174.18, 163.97, 156.25 (5C, *C*O), 139.63, 139.18, 135.67 (3C, 3*Cq*$_{arom}$), 125.54, 121.14, 116.16 (3C, *C*H$_{arom}$), 87.99 (1C, *C*H8b), 70.65, 70.41, 69.75, 69.68 (4C, *C*H_2O), 61.75, 60.22 (2C, *C*HNH), 55.49 (1C, *C*HS), 40.45 (1C, *C*H_2S), 39.71, 39.36 (2C, *C*H_2NH), 37.65 (1C, *C*H3a), 37.33 (1C, *C*H_2), 35.91 (1C, *C*H_2CO), 35.77 (1C, *C*H_2CO), 28.01, 27.90, 25.50 (3C, *C*H_2); Anal. calcd. for $C_{28}H_{39}O_7N_5S$-H_2O: C, 55.26; H, 6.80; N, 11.51 found: C, 55.35; H, 7.03; N, 11.17%.

tert-Butyl _N_-(2-{[3-((_E_)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(_SR_) furanyl]oxy}methylidene)-2-oxo-3,3a(_RS_),4,8b(_SR_)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl)carbamate *rac*-**18**

Protected Boc-amino-GR24 *rac*-**8** (150 mg, 0.36 mmol) was dissolved in a mixture of trifluoro acetic acid (1mL) and dichloromethane (10 mL) and stirred at room temperature for 3 h. When TLC analysis indicated complete deprotection, solvents were evaporated *in vacuo*. The residue was suspended in dichloromethane (5 mL) and treated with triethylamine (300 μL). After washing with satd. NaCl (1×) and drying ($MgSO_4$), volatiles were evaporated and the free amine was dissolved in THF (10 mL). Then 1.05 equiv. of 2,3,4,5,6-pentafluorophenyl 2-[(*tert*-butoxycarbonyl)amino]acetate (BocGlyOPfP, 130 mg, 0.38 mmol) and 1.05 equiv. of hydroxy benzotriazole (HOBT, 52 mg, 0.38 mmol) were added and the mixture was stirred at room temperature for 3 h. THF was removed *in vacuo* and the residue was purified over silicagel (ethyl acetate/hexane 3/1) to give *rac*-**18** as a white solid (161 mg, 95%) that was recrystallized from dichlomethane/ethyl acetate. Mp: 206-208 °C. ^{1}H NMR (300MHz,$CDCl_3$): δ 1.47 (s, 9H, 3 × CH_3 tBu), 2.05 (s, 3H, CH_3 D-ring), 3.04 (dd, 1H, $J_{4,\ 3a,\ cis}$ = 2.6 Hz, ^2J = 16.8 Hz, H4), 3.36 (dd, 1H, $J_{4,\ 3a,\ trans}$ = 9.3 Hz, ^2J =16.8 Hz, H4), 3.93 (m, 2H, H3a + CH_2 gly), 5.29 (bs, 1H, NH), 5.90 (d, 1H, J = 7.9 Hz, H8b), 6.18 (m, 1H, H2'), 6.97 (m, 1H, H3'), 7.17 (d, 1H, J = 8.2 Hz, H5), 7.49 (d, 1H, $J_{3a,\ 6}$ = 2.4 Hz, H6'), 7.52 (dd, 1H, J = 8.3 Hz, $^4J_{6,\ 8}$ = 1.5 Hz, H6), 7.62 (d, 1H, ^4J = 1.5 Hz, H8) 8.43 (bs, 1 H, NH). ^{13}C NMR ($CDCl_3$): δ 11.4 (CH_3 D-ring), 29.0 (CH_3 tBu), 37.6 ($CH_2$4), 39.9 (CH3a) 46.6 (CH_2 gly), 81.3 (Cq tBu), 86.5 (CH8b), 101.4 (CH2'), 113.9 (Cq C-ring), 118.4, 123.1, 126.3 (3 x CH_{arom}), 136.6, 137.7, 139.2, 140.2 (3 x Cq_{arom} and Cq D-ring), 141.7 (CH3'), 151.6 (CH6'), 153.4 (C=O carbamate), 168.6 (C=O gly), 170.9, 171.9 (2 x C=O lactones). IR (KBr): ν (cm^{-1}) 3346, 3282 (NH), 1788, 1742, 1721, 1682 (4 × C=O, C=C), 1183 (lactone). MS [FAB *m/z*, rel. intensity (%)]: 493 ($[M + Na]^+$, 54.3), 470 ($[M]^+$, 12.4), 371 ($[C_{19}H_{19}O_6N_2]^+$, 59.3), 97 ($[C_2H_5O_2]^+$, 100). Anal. calcd. for $C_{24}H_{26}O_8N_2$: C, 61.27; H, 5.57; N, 5.95 found: C, 61.06; H, 5.43; N, 5.93%.

***tert*-Butyl *N*-(2-{[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*S*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl)carbamate (-)- 18**

Compound (-)-**18** was prepared in the same way as described for *rac*-**18** starting from carbamate (-)-**8** (200 mg, 0.48 mmol). Yield: 97%. Recrystallization from dichloromethane/ethyl acetate gave a white fluffy solid. Mp: 189-190˚C. $[\alpha]^{22}_D$ = -298.9˚ (c=0.1, CH_2Cl_2). Anal. calcd. for $C_{24}H_{26}O_8N_2$: C, 61.27; H, 5.57; N, 5.95 found: C, 61.03; H, 5.55; N, 5.92%. All other analytical data were the same as reported for its racemate rac-**18**.

4-[({(2-{[3-((*E*)-1-{[4'-Methyl-5'-oxo-2',5'-dihydro-2'(*S*) furanyl]oxy}methylidene)-2-oxo-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl]amino}carbothioyl)amino]-2-[6(dimethylamino)-3-(1,1-dimethylammonio)-3H-9-xanthenyl]benzoate (-)-**19**

The amine protecting group was removed from carbamate (-)-**18** (16 mg, 0.034 mmol) by stirring for 1 hour at room temperature in a mixture of dichloromethane (2 mL) and trifluoro acetic acid (0.25 mL). Subsequently the mixture was concentrated under reduced pressure and the residue was dissolved in dichloromethane (0.5 mL) and triethylamine (100 μL) was added to liberate the free amine. Tetramethyl rhodamine isothiocyanate (15 mg, 0.034 mmol) was dissolved in DMF (2 mL) and added to the solution of deprotected (-)-**18.** The desired thiourea linkage was formed instantly, as indicated by TLC analysis. After stirring for 1 hour at room temperature, solvents were evaporated *in vacuo*, and the residue was purified by flash chromatography over silica gel (10%-50% methanol in dichloromethane) yielding a bright pink solid (14 mg, 53%). mp: >300°C. $[\alpha]^{22}_D$ = -270.6° (c=0.03, H_2O). ^{1}H NMR (300MHz, DMSO-d_6): δ 1.92 (s, 3H, CH_3 D-ring), 2.88 (m, 1H, H4), 2.94 (s, 12H 4 CH_3TRITC), 3.30 (m, 1H, H4), 3.94 (m, 1H, H3a), 4.30 (bs, 2H, CH_2gly), 5.97 (d, 1H, J = 7.9 Hz, H8b), 6.49-6.58 (m, 6H, NH thiourea + 4 H_{arom}TRITC), 6.69 (m, 1H, H2'), 6.82 (d, 1H, J = 8.3 Hz, H_{aroma}TRITC), 7.14 (d, 1H, J = 8.3 Hz, H_{aroma}TRITC), 7.22 (d, 1H, J = 8.3 Hz, H5), 7.40 (m, 1H, H3'), 7.46 (d, 1H, J = 8.3, H6),

7.70 (d, 1H, $^4J_{3a,\,6'}$= 2.4 Hz, H6') 7.75 (bs, 1H, H8), 7.80 (d, 2H, J = 7.9 Hz, H_{arom}TRITC), 7.89 (d, 1H, J = 8.7 Hz, H_{arom}TRITC), 10.2 (s, 1H, NH_{arom}). ^{13}C NMR (DMSO-d_6): δ 10.2 (CH_3 D-ring), 36.3 ($CH_2$4), 38.6 (CH3a), 39.8 (CH_3TRITC), 47.4 (CH_2 gly), 85.2 (CH8b), 101.3 (CH_2'), 106.3 (2 x CqTRITC), 111.7(CqC-ring), 97.9, 108.3, 113.8, 116.2, 121.1, 125.5, 120.6, 121.0, 124.9, 125.5, 127.4, 128.3 (12 x CH_{arom}), 133.7 (CqD-ring), 137.3, 138.0, 139.7 (3 x $Cq_{arom,}$ GR24), 143.2 (CH3'), 151.8, 152.1 (6 x CqTRITC), 152.8 (CH6'), 153.8 (Cq-COO^-), 167.9, 168.4, 170, 170.6 (4C, C=O), 180.1 (1C, C=S). IR (KBr): ν(cm^{-1}) 3422 (CO<u>OH</u>), 3299 (NH), 2989 (CH_{arom}), 1783, 1749, 1680 1648 (5 x C=O, C=C), 1536 (amide II). MS [FAB *m/z*, rel. intensity (%)]: 836 ($[M + Na]^+$, 7.3), 814 ($[M + H]^+$, 13.7), 414 ($[C_{20}H_{18}O_6N_2S]^+$, 100).

9H-9-Fluorenylmethyl-*N*-[2-{[3-((*E*)-1-{[4'-Methyl-5'-oxo-2',5'-dihydro-2'(*SR*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*RS*),4,8b(*SR*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-1-({[(acetylamino)methyl]sulfanyl}methyl)-2-oxoethyl]carbamate] ***rac*-20**

Racemic, slow moving Boc-amino-GR24 diasteroisomer ***rac*-8** (829 mg, 2.0 mmol) was deprotected by stirring for 1 hour at room temperature in a mixture of dichloromethane (9 mL) and trifluoro acetic acid (1 mL). Then solvents were evaporated under reduced pressure. The residue was dissolved in dichloromethane (10 mL) and triethylamine (1 mL) was added to liberate the free amine. The mixture was washed with brine (1 x), dried ($MgSO_4$) and concentrated. After dissolving the residue in THF (10 mL), 1.1 equiv. of Fmoc-and Acm-protected pentafluorophenyl ester of cysteine (FmocCys(Acm)OpfP, 1.28 g, 2.2 mmol) and 1.1 equiv. of hydroxy benzotriazole (HOBT, 300 mg, 2.2 mmol) dissolved in THF (3 mL) were added. The mixture was stirred at room temperature for 3 hours. THF was removed *in vacuo* and the residue was dissolved in ethyl acetate (20 mL), washed with brine (1x), dried ($MgSO_4$) and concentrated *in vacuo*. Silicagel chromatography (dichloromethane/methanol 15/1) gave ***rac*-20** as a white solid (1.39 g, 98%). An analytically pure sample was obtained by recrystallization from ethyl acetate/diisopropyl ether. Mp: 204-206°C. ^{1}H NMR (300 MHz, DMSO): δ 1.86 (s, 3H, CH_3 Acm), 1.92 (s, 3H, CH_3 D-ring), 2.77-2.99 (m, 3H, CH_2 cys + H4), 3.30 (m, 1H, H4),

3.94 (m, 1H, H3a), 4.21-4.40 (m, 8H, CH cys + CH_2 cys + CH Fmoc + CH_2 Fmoc + CH_2 Acm), 5.98 (d, 1H, J = 7.9 Hz, H8b), 6.70 (m, 1H, H2'), 7.24 (d, 1H, J = 8.3 Hz, H5), 7.30-7.90 (m, 10H, NHcys + H3' + 8H_{arom}Fmoc), 7.70 (d, 1H, $^4J_{3a,\,6'}$ = 2.3 Hz, H6'), 7.74 (d, 1H, J = 8.3 Hz, H6), 7.87 (bs, 1H, H8), 8.58 (t, 1H, J = 6.2 Hz, NHAcm), 10.10 (s, 1H, NHarom). ^{13}C NMR (DMSO): δ 10.2 (CH_3D-ring), 22.6 (CH_3Acm), 32.3 (CH_2cys), 36.3 ($CH_2$4), 38.6 (CH3a), 40.2 (CH_2Acm), 46.6 (CHFmoc), 65.8 (CH_2 Fmoc), 85.2 (CH8b), 101.3 (CH2'), 111.7(CqC-ring), 116.4, 121.1, 125.5 (3 x CH_{arom}GR24), 120.1, 125.3, 127.1, 127.6 (8 x CH_{arom}Fmoc), 133.7 (CqD-ring), 137.4, 138.0, 139.7 (3 x $Cq_{arom,}$ GR24), 140.7, 143.8 (4 x CqFmoc), 143.2 (CH3'), 152.8 (CH6'), 156.0 (C=O Fmoc), 169.4, 169.8, 170.7, 170.8 (4 x C=O). IR (KBr): ν (cm^{-1}) 3327 (NH), 3066 (CH_{arom}), 2942 (CH_{aliph}), 1795, 1770, 1736, 1709, 1695, 1679 (5 x C=O, C= C), 1535 (amide II). MS [FAB *m/z*, rel. intensity (%)]: 732 ($[M + Na]^+$, 28.2), 710 ($[M + H]^+$, 15.3). Anal. calcd. for $C_{38}H_{35}O_9N_3S$: C, 64.30; H, 4.97; N, 5.92; S, 4.52 found: C, 63.83; H, 4.94; N, 5.70; S, 4.26.

_N_-1-(2-{[3-((*E*)-1-{[4'-Methyl-5'-oxo-2',5'-dihydro-2'(*SR*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*RS*),4,8b(*SR*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl)-4-azido-2-hydroxy-benzamide *rac*-**23**

Carbamate *rac*-**8** (39 mg, 0.083 mmol) was dissolved in a mixture of dichloromethane (2 mL) and trifluoro acetic acid (0.25 mL) and stirred at room temperature for 2 hours. When TLC indicated complete removal of the Boc protective group, solvents were evaporated *in vacuo*. The residue was dissolved in THF (2 mL) and triethylamine (100 μL) was added which was immediately followed by the addition of 1:1 equiv. of azido salicylic acid succinimidyl ester **22** (25 mg, 0.090 mmol), dissolved in THF (0.5 mL). The reaction takes place instantaneously, as was indicated by TLC analysis, and a white solid appeared in the solution. According to TLC analysis, this solid was excess ester **22,** which was removed by filtration. THF was evaporated *in vacuo*. The residue was dissolved in ethyl acetate, washed with aqueous $NaHCO_3$ (5%) to remove N- hydroxy succinimide, dried ($MgSO_4$) and concentrated. Upon concentration, *rac*-**23** (44 mg, 99%) was obtained as white solid, which was recrystallized from ethyl acetate/hexane. Mp: 200˚C. ^{1}H NMR (300MHz, DMSO): δ 1.92 (s, 3H, CH_3 D-ring), 2.87 (dd, 1H, $J_{4,\,3a\,cis}$ = 2.5 Hz, ^{2}J= 16.7 Hz, H4), 3.30

(m, 2H, H4 + OH), 3.94 (m, 1H, H3a), 4.11 (m, 2H, CH_2 gly), 5.97 (d, 1H, J = 7.9 Hz, H8b), 6.63 (d, 1H, ^{4}J = 2.1 Hz, -CN_3C<u>H</u>COH-), 6.70 (m, 1H, H2'), 6.70 (dd, 1H, J_1 = 8.6 Hz, ^{4}J= 2.1 Hz, -CN_3CHCH-), 6.97 (m, 1H, H3'), 7.23 (d, 1H, J = 8.3 Hz, H5), 7.49 (d, 1H, J= 8.3 Hz, H6), 7.70 (d, 1H, $^4J_{3a,\ 6'}$= 2.3 Hz, H6'), 7.79 (bs, 1H, H8). 7.95 (d, 1H, J = 8.6 Hz, CN_3CHC<u>H</u>-), 9.41 (bs, 1H, NH gly), 10.18 (S, 1H, NH_{arom}). ^{13}C NMR (DMSO): δ = 10.2 (CH3 D-ring), 36.4 ($CH_2$4); 38.5 (CH3a), 43.0 (CH_2 gly), 85.2 (CH8b), 101.3 (CH2'), 111.8 (Cq_{arom} C-ring), 112.7 (Cq_{arom}), 107.0, 110.0, 116.3, 121.0, 125.5, 130.3 (6 x CH_{arom}), 133.7 (CqD-ring), 137.3, 138.1, 139.7 (3 x $Cq_{arom,}$ GR24), 143.2 (CH3'), 144.5 (Cq_{arom}-N_3), 152.8 (CH6'), 160.7 (Cq_{arom}-OH), 167.3, 167.9, 170.6, 170.8 (4 x C=O). IR (KBr): ν (cm^{-1}) 3575 (OH), 3315 (NH), 1780, 1742, 1728, 1700, 1689 (4 x C=O, C=C). MS [FAB *m/z*, rel. intensity (%)]: 554 ($[M + Na]^+$, 24.6), 532 (($[M + H]^+$, 32.3), 97 ($[C_5H_5O_2]^+$, 51.1). Anal. calcd. for $C_{26}H_{21}O_8N_5$: C, 58.76; H, 3.98; N, 13.18 found: C, 57.99; H, 3.99; N, 12.94%.

X-ray crystallography. Crystals of **(+)-8,** suitable for X-ray diffraction studies, were obtained by slow evaporation from 2-methyl-2-butanol. A single crystal was mounted in air on a glass fiber. Intensity data were collected at room temperature. An Enraf-Nonius CAD4 single crystal diffractometer was used, CuK_α radiation, θ-2θ scan mode. Unit cell dimensions were determined from the angular setting of 25 reflections. Intensity data were corrected for Lorentz and polarization effects. Semi-empirical absorption correction (ψ-scans) was applied. The structure was solved by the program CRUNCH and was refined using standard methods (refinement against F^2 of all reflections with SHELXL-97) with anisotropic parameters for the non-hydrogen atoms. All hydrogens were initially placed at calculated positions and were freely refined subsequently. Relevant numerical data were collected: table 2.

Table 2 Crystal data and Structure refinement for slow moving BocNH-GR24 diastereoisomer **(+)-8**

Empirical formula	$C_{22}H_{23}NO_7$	Index range	$0 \le h \le 11$, $0 \le k \le 15$, $0 \le k \le 21$,
Formula weight	413.41		
Crystal colour	Transparent colourless	Reflections collected/unique	2282/2282
Crystal shape	Regular rod		
Crystal size	0.42 × 0.12 × 0.11 mm	Reflections observed	1967($[I_0>2\sigma(I_0)]$)
Temperature	293(2) K	Absorption correction	semi-empirical from Ψ–scans
Radiation/wave length	CuK_α (graphite monochrom.)/1.54184 Å	Range of relat. transm. factors	1.055 and 0.960
Crystal system/space group	Orthorhombic/$P2_12_12_1$	Refinement method	full-matrix least squares on F^2
Unit cell dimensions (25 reflections 22.177<θ<45.115)	a = 9.3201(4) Å b = 12.5782(4) Å c = 17.9679(4) Å	Computing	SHELXL-97[31]
Volume	2106.38(11) $Å^3$	Data/restraints/parameters	2282/0/364
Z/calculated density	4/1.304mg/m^3	Goodness-of-fit on F^2	1.048
absorption coefficient	0.815 mm^{-1}	SHELXL-97 weight parameters	0.077600 and 0.140500
diffractometer/scan	Enraf-Nonius CAD4/θ–2θ	Final R indices [$I>2\sigma(I)$]	R_1=0.0398, wR_2=0.1093
F(000)	872	Final R indices (all data)	R_1=0.0485, wR_2=0.1169
θ range for data collection	4.29 to 69.94	Extinction coefficient	0.0023(4)
		Largest diff. peak and hole	0.192 and –0.178e. $Å^3$

Bioassays

Plant material. Seeds of *Striga hermonthica* (Del.) Benth. Were collected from Sorghum (Sorghum bicolor (L.) Moench) on Gezira Research station, Sudan in 1994. The seeds were stored in glass vials in the dark at room temperature until use in germination tests.
Preparation of test solutions. A compound to be tested was weighed out very accurately to the amount of 1.0 mg, dissolved in 5 mL acetone p.a. and diluted with dematerialized water to 50 mL. These stock solutions of approximately 10^{-4} mol L^{-1} (the exact concentration depending on the molecular mass of the compound used) were further diluted with dematerialized water to obtain test solutions with concentrations ranging from 2×10^{-5} and 2×10^{-7} mol L^{-1} . All solutions were prepared directly before use.
Bioassays. All bioassays were performed at the Department of Organic Chemistry University of Nijmegen, The Netherlands in 1999 and 2001. For surface sterilization all seeds were exposed for 5 minutes to 50% (v/v) aqueous solutions of commercial bleach (2% hypochlorite). Subsequently, the seeds were thoroughly rinsed with demineralized water and air-dried. For conditioning the seeds were spread on glass fiber filter paper disks (8 mm diameter, approximately 60-100 seeds per disk) in Petri dishes, wetted with

demineralized water and stored in the dark at 30°C. Thereafter the conditioning water was removed and conditioned seeds were placed in new Petri dishes and exposed to test solution. After incubation for 24 hours in the dark at the indicated temperatures the percentages of germination seeds were determined under a microscope. Seeds were considered to be germinated if the radicle protruded through the seed coat. In each test series an aqueous solution of 0.1% acetone was included as a negative control. For full details of the bioassays, see Mangnus *et al.*[37]

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CHAPTER 6

Detection of a specific protein binding site for germination stimulants in the seeds of *Striga hermonthica* using a biotinylated strigolactone

Abstract: Strigolactones are highly potent germination stimulants for seeds of the parasitic weeds *Striga* and *Orobanche* spp. The induction of seed germination is thought to proceed via a receptor-mediated mechanism. For the isolation and identification of this receptor using affinity purification by streptavidin beads, a biotin labelled strigolactone (BioGR-24) is required. The synthesis of biotinylated GR24 and the negative control BioGR24N was described in chapter 5. In this chapter, BioGR24 and BioGR24N were used to detect and isolate a binding protein. The presence of a specific strigolactone binding protein (SLBP) in *Striga* seed insoluble membrane fractions was demonstrated by a dot-blot analysis. Preliminary results with SDS-PAGE showed an enrichment of a 60 kDa protein isolated from these fractions by affinity purification.

6.1 Introduction

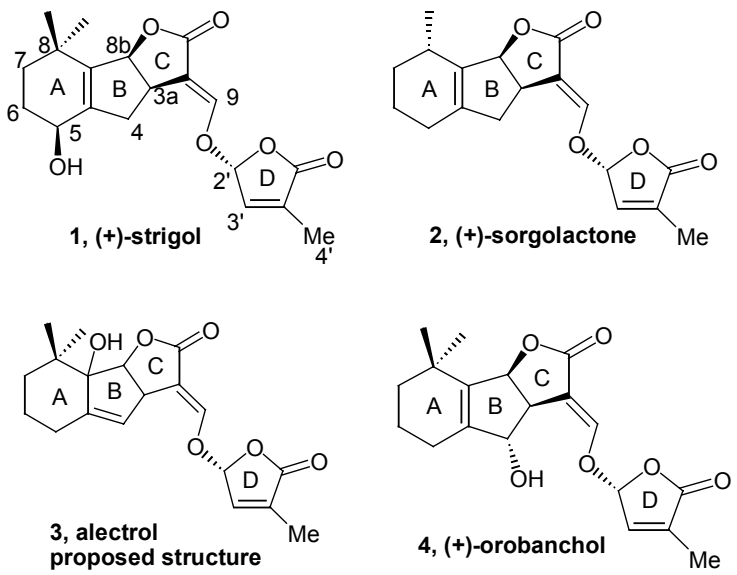
Parasitic weeds belonging to the genera *Striga* and *Orobanche* severely reduce yields of economically important food crops, such as maize, sorghum, millet and rice, in some cases more than 50%.[1,2] The lifecycle of these parasitic weeds is extremely well adapted to their host plants.[3,4] Seeds germinate in the soil, after a preconditioning period by exposure to moisture at an appropriate temperature, only in response to a specific chemical germination stimulant exudated by host plant roots. Only seeds within the host root rhizosphere will germinate. The parasite seedling radicle only grows a few mm and must contact a host root to ensure its survival. Upon contact with the host root, the radicle develops a specialized parasitic-plant organ, the haustorium. This attaches to the root, penetrates the epidermis and cortex, and ultimately establishes connections to the host vascular system.[5,6,7] In this manner the parasite can withdraws water and nutrients from the host.

Several naturally occurring germination stimulants (strigolactones), namely, (+)strigol,[8] (+)-sorgolactone,[9] (+)-orobanchol[10] and alectrol[11] (Figure 1) were isolated from host plants. The

Abbreviations: BioGR24, biotin labeled GR24; SDS-PAGE,sodium dodecyl sulfate poly acryl amide gel electrophoresis; PMSF, Phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, 1,4-Dithio-DL-threitol; PBS: phosphate buffer solution; BSA, bovine serum albumin; HRP; horseradish peroxidase

biological activity studies of these stimulants revealed that they are active at a low concentration (10^{-8}-10^{-10}M).[12,13,] Furthermore, structure-activity relationship studies have clearly revealed the importance of the absolute stereochemical configuration of strigolactones for the optimal germination activity[13b]. It was found that stereoisomers of strigolactones possessing the same absolute configuration at the CD part as in the natural stimulant exhibit the highest germination stimulatory activity[13b,14,15,] (see also chapter 2). It may be therefore concluded that the germination stimulants are selectively recognized by the seeds of the parasitic weeds. On the basis of this observation it has been hypothesized that the induction of *Striga* and *Orobanche* seeds germination proceeds via a receptor mediated mechanism.[16] It is also relevant to mention that the bioactiphore of the stimulants resides in the CD-part of the molecule whereby the D-ring is a prerequisite.[16,17,18] Although that a molecular mechanism has been proposed for the initiation of the germination,[18] the true mode of action of the stimulant is unknown. In this chapter, the first attempt to identify and isolate the postulated protein receptor from the seeds of the parasitic weeds *Striga hermonthica* (Del) Benth. is described. Detailed knowledge of the receptor protein is essential for the better understanding of the initial stages of the germination process on the molecular level. Such information may also contribute to design and develop better and new methods for the control of parasitic weed pests.

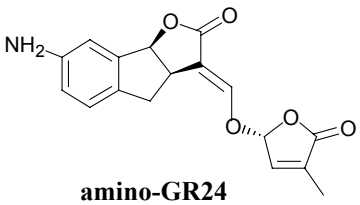
Figure 1



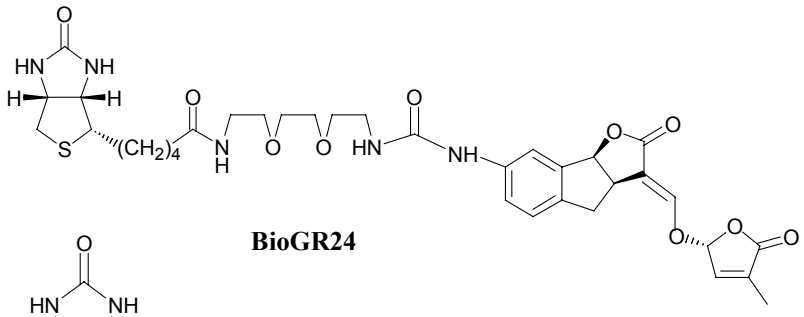
6. 2 Results

For the purpose of identifying and isolating of the strigolactone receptor, the biotin streptavidin affinity methodology was used. A suitable synthetic germination stimulant for attaching a biotin affinity tag is amino-GR24 (Figure 2). It has been demonstrated that introduction of substituents in the A-ring of GR24 hardly affects the biological activity. Therefore, the biotin label can be attached to this A-ring. The synthesis of biotinylated GR24 (BioGR24) (Figure 2) having a hydrophilic polyethylene glycol (PEG) linker has been described in chapter 5. This PEG linker was chosen in order to increase the water solubility of the biotin derivative. A biotinylated analogue lacking the D-ring was also prepared. This compound (BioGR24N) (Figure 2) serves as a negative control to exclude non-specific interaction. It was shown that BioGR24 indeed retained its germination stimulatory activity (Chapter 5). It is important to note that the compounds shown in Figure 2 all have the natural configuration at the respective stereogenic centres. This is necessary to warrant optimal response to the seeds of parasitic weeds.

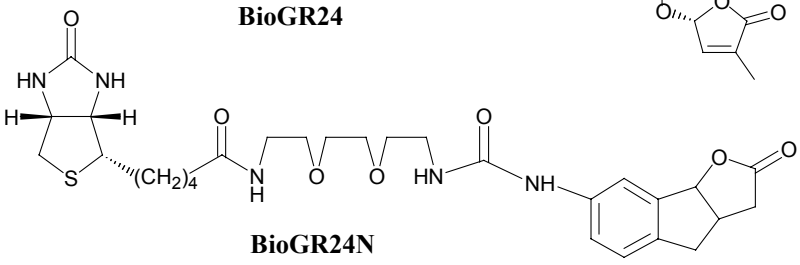
Figure 2



amino-GR24



BioGR24



BioGR24N

For the protein extraction from the seeds the protocol shown in Figure 3 was followed. The preconditioned seeds of *Striga hermonthica* were incubated with BioGR24 or water (group A seeds and group B seeds, respectively). Then the frozen seeds were grounded and extracted with buffers of increasing hydrophobicity and chaotropic potential. The resulting four extracts were then incubated with BioGR24, BioGR24N and water, respectively.

Figure 3: Protocol for the protein extraction from seeds of *Striga hermonthica*:

Incubated of pre-conditioned seeds with BioGR24 (group A) or water (group B) for 12h	
Grinding of the frozen seeds (liq. N_2) of both populations.	
Extraction with Buffer 1 (only salts +buffer)	Extract 1
Extraction with Buffer 2 (Triton X-100)	Extract 2
Extraction with Buffer 3 (urea+CHAPS)	Extract 3
Extraction with Buffer 4 (Laemli buffer+heating)	Extract 4
Extracts were incubated with BioGR24 or BioGR24N for 30 min followed by dot-blot assay.	

Buffer 1: 10 mM Tris HCl pH = 8; 1 mM EDTA; 1 mM PMSF.

Buffer 2: 4% triton-X; 10% glycerol; 10 mM Tris HCl pH=8; 1 mM PMSF

Buffer 3: 8M urea; 4% CHAPS; 100 mM DTT; 10 mM Tris buffer pH 8

Buffer 4: 2% SDS; 100 mM DTT; 30% glycerol; 100 mM Tris HCl pH=6.8.

After spotting on a filter and washing several times, the species bound to biotin were detected with streptravidin-horse radish peroxidase (HRP). The spot-blot assays revealed binding of BioGR24 in the two most chaotropic extracts of *Striga* seed that had been prestimulated with BioGR24 (Figure. 4a). The negative control BioGR24N, did not show binding in any of the four extracts (Figure 4a, lowest panels). In a competition experiment (Figure 4c) performed by incubating extract 4 with a mixture of BioGR24 and a 100-fold molar excess of GR24, the binding with BioGR24, as expressed by the chemoluminescence signal, was significantly lowered (Figure. 4c). In subsequent experiments the affinity purification technique was applied on extracts 3 and 4 from both populations (group A and B). These extracts were incubated with BioGR24 ($6x10^{-7}$ M solution) and then treated with paramagnetic streptavidin beads. After denaturation by boiling the beads with SDS-Laemli buffer, the solution was analysed on 12% SDS-PAGE. The result is shown in Figure 5. A molecular mass marker was used to determine the molecular weight of the tagged protein. The SDS-PAGE shows that streptavidin clearly retains a protein of ca 60 kDa from extract 3 and 4 obtained from seeds

that had been pretreated with BioGR24 (group A seeds), In contrast, in the corresponding extracts obtained from the group B seeds, no protein could be detected (Figure 4B):

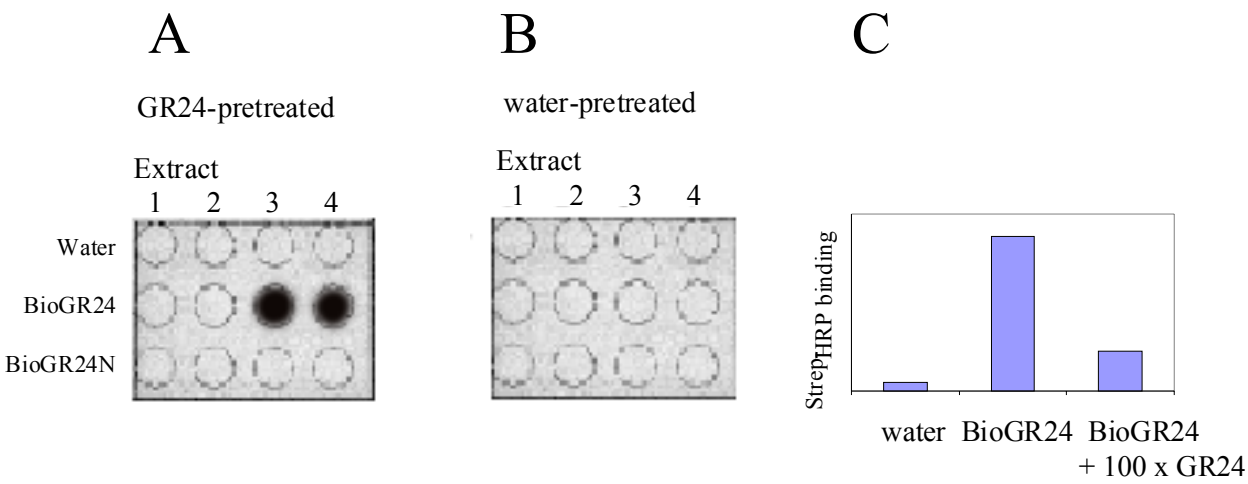


Figure 4 **(A)** Chemoluminescent detection of bound BioGR24 in a dot-blot analysis. Extract 3 and 4 from BioGR24 pretreated seeds showed a signal after incubation with additional BioGR24. **(B)** Chemoluminescent detection of bound BioGR24 in a dot-blot analysis of extracts from water treated seeds (group B) shows no signal. **(C)** Competition of BioGR24 by unlabelled GR24: Chemoluminiscence signal from biotin-bound HRP streptavidin is significantly lowered by the addition of 100 fold molar excess of unlabelled GR24.

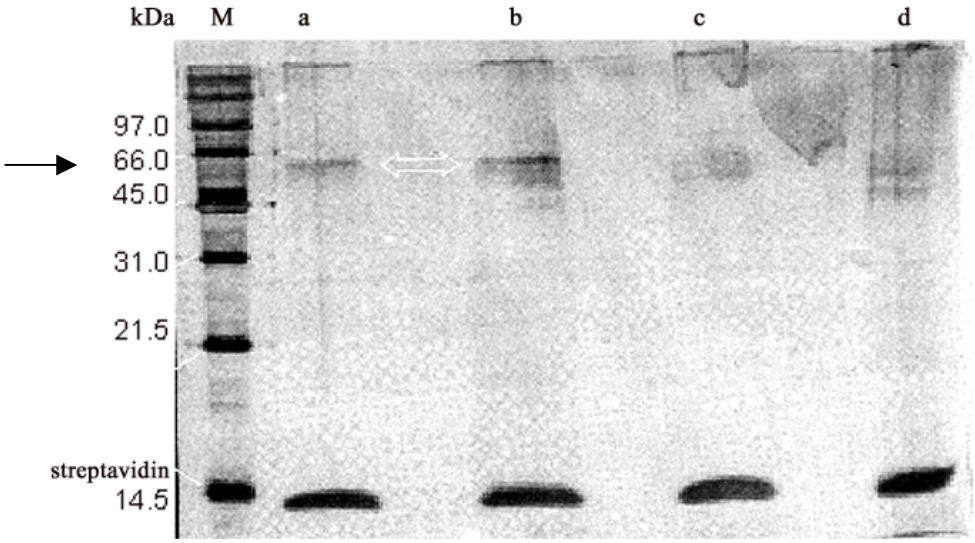


*Figure 5***.** SDS-PAGE of extract 4 (lane a) and extract 3 (lane b) from group A seeds, after affinity purification with streptavidin beads. Similarly, extract 4' (lane c) and 3' (lane d) from group B seeds after affinity purification with streptavidin beads. All lanes were stained with silver. Molecular mass marker: 60 kDa for the SLBP Group A seeds: pretreated with BioGR24 before grinding; group B seeds: pretreated with water only.

6.3 Discussion

Parasitic weeds of the genus *Striga* reduce yields of economically important food crops in tropical areas of the southern hemisphere. The lifecycle of this parasitic weed is intimately tuned to the presence of a suitable host plants and germination of their seeds is entirely controlled by metabolites exuded by the host roots, called germination stimulants (strigolactones). Germination is one of the early stages in the parasitic life cycle that can be targeted for genetic control. The aim of this work is isolation and identification of the receptor for germination stimulants (strigolactones). The importance of this work is multi-faceted. Firstly, a contribution to the understanding of the initial stages of the germination process of these parasitic weed seeds on the molecular level. This will allow developing new methods for parasitic weed control, which at present are not satisfactory. Parasitic weed control is important especially in developing countries, where the food supply is fully based on the local agriculture.[19] Secondly, a detailed knowledge of plant-weed interaction on the molecular level can be obtained.

The protein extraction of *Striga hermonthica* grounded seeds was performed with four buffers of increasing chaotropic potential (Figure 3). In the first extraction water-soluble proteins are extracted, in the second the membrane-associated proteins and in the third and fourth extraction membrane-bond proteins are collected. Two sets of seeds were subjected to this procedure, *viz* the group A seeds that were pretreated (stimulated) with BioGR24 for 12h before grinding and group B seeds that were pretreated with water only. The resulting extracts were incubated with BioGR24 or BioGR24N as the negative control. The dot blot analysis of the thus treated extracts revealed the presence of (a) binding protein(s) in the two most chaotropic extracts derived from the group A seeds. This observation strongly suggests the presence of a specific binding for BioGR24. The negative control did not show any binding. This absence of any binding with BioGR24N, which lacks the essential D-ring, substantiates that the binding of BioGR24 is highly specific. It was also ascertained that streptavidin itself did not produce a signal, nor did biotin or any endogenous peroxidase activity. Furthermore, the competition experiment using BioGR24 and a hundred fold excess of GR24, confirms the specifity of the binding. This strigolactone binding protein (SLBP), isolated from the membrane fractions of *Striga* seeds, may serve as a receptor for the germination stimulants. Unexpectedly, practically no protein was observed in the most chaotropic extracts derived from the seeds that had been treated with water only (group B seeds). This remarkable finding can be accounted for by assuming that strigolactones up-regulate the expression of their

receptor during the pre-incubation of the seeds with BioGR24. This phenomenon of up-regulation shows some resemblance with the observations that the putative ethene receptor NR in tomato[20] and ETR2 in Arabidopsis[21] exhibit enhanced expression upon ethene treatment. In animal systems, several growth factors were shown to induce expression of their receptor.[22] Here, ligand-mediated receptor up-regulation is believed to constitute a positive feedback mechanism that could serve to potentiate autocrine stimulation of growth. Similarly, *S. hermonthica* seeds, once sensing the presence of a germination stimulant, initiate or enhance the expression of strigolactone receptor, thus corroborating the signaling role of strigolactones and their receptors. This positive feed-back phenomenon of the SLBP may serve to increase the sensitivity to the stimulant and to alter the physiology of the embryo leading to germination of the embryo.

The isolation of the SLBP from the protein extracts of the group A seeds was performed by affinity purification using streptavidin beads. The isolated protein had a molecular mass of 60kDa. As expected the extracts obtained from the group B did not show any protein in the SDS-PAGE. The affinity purification nicely complements the conclusion derived from the protein analysis using the dot blot technique.

In conclusion, the results described in this chapter demonstrate the presence of a SLBP in the seeds of parasitic weeds with a molecular mass of ca 60kDa. This is the first time that such a binding protein for strigolactones has been detected and characterized. It should be mentioned that only in a few cases receptor proteins have been isolated from plant material, especially seeds. In the majority of cases receptors are identified by genetic studies, involving the isolation of genes**,** that block the signal transduction of certain hormones when knocked down.[20]

The detection and characterization of the SLBP, as described in this chapter, is highly relevant for further research to unravel the lifecycle of parasitic weeds; immuno-electron microscopy can be applied for affinity studies of ligand-receptor interaction using BioGR24. For signal amplification, colloidal gold conjugated to streptavidin can be used. The power of electron microscopy for this purpose is manifold: overview of 300μm-sized structures as well as detailed nanometer resolution images of e.g. colloidal gold can be obtained. Preliminary FESEM (Field Emission Scanning Electron Microscopy) studies to identify the plant tissues where the germination starts were carried out with imbibed *Striga hermonthica* seeds (see appendix).

Sequencing of parts of the protein will allow isolation the cDNA encoding the receptor, and further underscore its binding properties by heterologous expression experiments in yeast and plant cells. Cells expressing such a receptor are useful to select antagonistic compounds from large libraries, as has for instance been reported for prostate cancer cells[23]. Such antagonistic compounds could possibly be used in controlling the parasitic weed pests in the field. The results described here open a new window in parasitic weed research.

6.4 Experimental

Plants material:

Seeds of *Striga hermonthica* (Del.) Benth. were collected from Sorghum (Sorghum bicolor (L.) Moench) on Gezira research Station, Sudan. The seeds were kindly supplied by prof. A. Babiker.

Protein Extraction:

Striga seeds (4 g, dry weight) were conditioned for 14 days at 30^0C in a closed Petri dish, containing 10 mL of sterilized demineralized water. Seeds were air-dried for 30 min and split in two populations. One population (group A) was treated (stimulated) with solution of BioGR24 (1 mL 10^{-5}M) for 12 h at 30^oC in the dark, while the other population (group B) underwent the same treatment with water. Subsequently**,** seeds from both populations were frozen with liquid nitrogen and ground as finely as possible using a mortar and pestle. The ground material (70 mg) was subjected to consecutive protein extraction with 1 mL of four extraction buffers. The first extraction was performed with Buffer 1 (10 mM Tris HCl pH = 8; 1 mM EDTA; 1 mM PMSF). The sample was sonicated on ice 6 times for 10 seconds (5 sec. rest, amplitude 16) and centrifuged for 5 min at 14,000g and 4^0C. The supernatant (extract 1) was collected and 2 mM of PMSF was added. The resulting pellet was extracted by sonication as with Buffer 1, but using 1 mL Buffer 2 (4% triton-X; 10% glycerol; 10 mM Tris HCl pH=8; 1 mM PMSF). After centrifugation, the supernatant (extract 2) was stored on ice, and the pellet was extracted with 1 mL Buffer 3 (8 M urea; 4% CHAPS; 100 mM DTT; 10 mM Tris buffer pH 8) by sonication in the same way. After centrifugation, the supernatant (extract 3) was stored on ice. The pellet was resuspended in 500 µl Laemli buffer (2% SDS; 100 mM DTT; 30% glycerol; 100 mM Tris HCl pH=6.8) and boiled for 5 min. After centrifugation, the supernatant was stored on ice (extract 4).

The relative concentration of protein in all four extracts was determined using the Bio-Rad Protein Assay.

Dot-blot

Bio-GR24 (1 μL of a 10^{-5} M solution) or Bio-GR24N (1 μL of a 10^{-5} M solution) was added to 2 μL of each of the four extracts. The extracts were incubated for 30 min at room temperature to allow binding. Subsequently, 200 μL of PBS was added and the respective extracts were loaded under vacuum on a SRC 96D Minifold I Dotblotter (Schleicher & Schuell, Dassel) containing nitrocellulose. The nitrocellulose sheet in the Dotblotter was washed three times with PBS, taken out of the Dotblotter and blocked for 30 min with PBS with 0.1% Tween 20 and 0.1% BSA), rinsed and incubated for 30 min with horseradish peroxidase-conjugated streptavidin (Boehringer, Mannheim) in PBS with 0.1%Tween, washed three times 5 min with PBS with Tween and detected by chemoluminiscence.
times 5 min with PBS with Tween and detected by chemoluminisence.
The competition experiment was performed with extract 4 in the same manner as described above, except that the extract was treated with 1μL BioGR24 admixed with 1μL of 10^{-4}M solution of GR24. The results of the dot-blot analysis are shown in Figure 4.

Affinity Purification

100 μl of extracts 3 and 4 of both seed populations (group A and group B) were incubated for 30 min. at room temperature with $6x10^{-7}$ M solution of BioGR24. Subsequently, 10 μl of paramagnetic streptavidin-coated beads (Dynal) suspension was added, and again incubated for 30 minutes. The resulting beads were washed three times with PBS and 0.1 % Tween and denatured by boiling with SDS-Leamli buffer for 10 min. The solution was then analysed on 12% SDS-PAGE, and stained with silver according to Rabilloud et al.

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Appendix

Electron Micrographs of *Striga hermonthica* seeds (Figure a-d). Preliminary CLSM (Confocal Laser Scanning Microscopy) and FESEM (Field Emission Scanning Electron Microscopy) studies were carried out in order to locate the site of the stimulant-receptor interaction in the seeds. The seeds were conditioned (in water) for 7 days and subsequently cryo-fractioned and coated with gold-palladium.

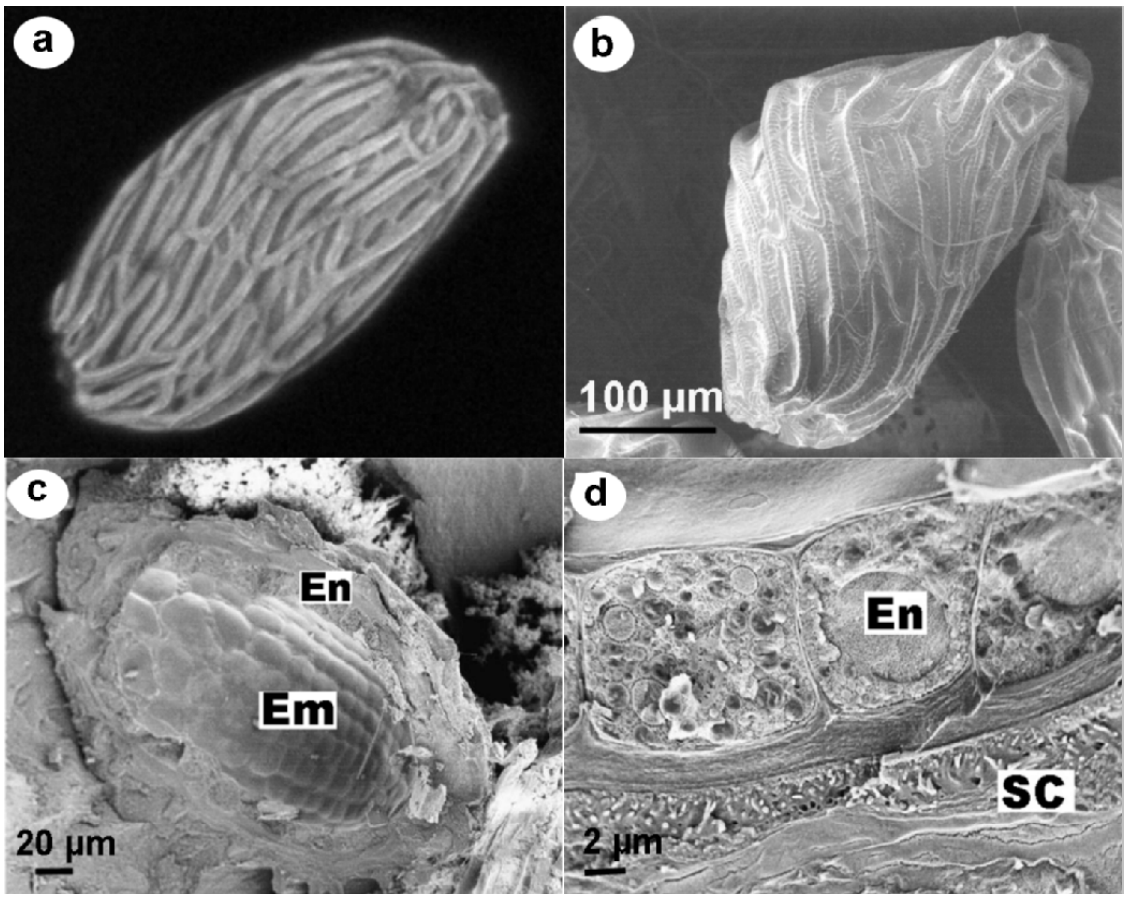


Figure a) CLSM of autofluorescence in a seed of *Striga hermonthica* obtained using 488 nm excitation wavelength on BioRad MRC-600 microscope.

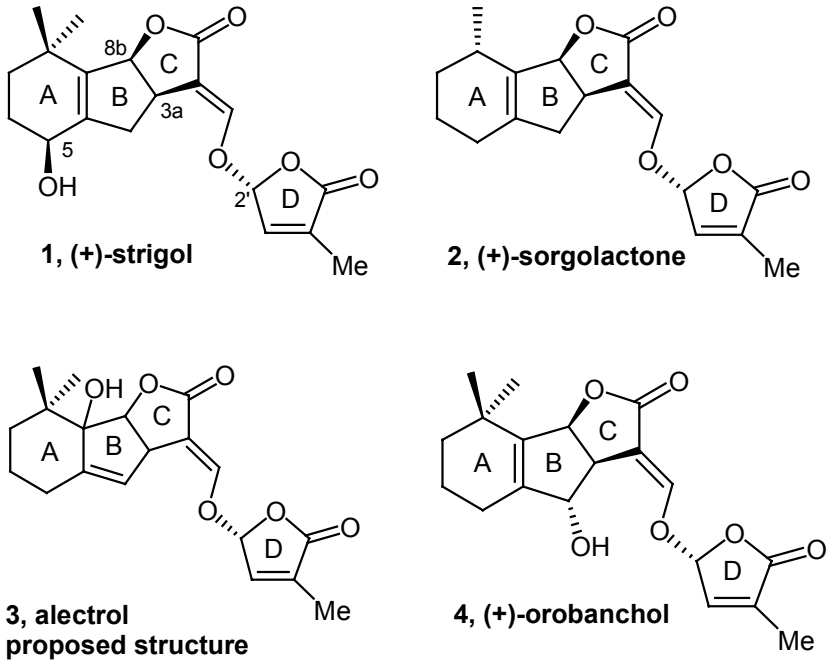
b-d) FESEM showing: (b) An overview of the seed surface. (c) The embryo (Em) and the endosperm layer (En) in fractioned seed. (d) Single cells of the endosperm layer and part of the seed coat (SC).

Summary

The angiosperms *Striga* (scorphulariaceae) and *Orobanche* (Orobanchaceae) are root parasitic plants, which have a devastating effect on their hosts. They both attack important food crops: *Striga* spp. preferentially attacks monocotyledonous crops such as maize, sorghum, millet and rice, in tropical and sub-tropical areas in Africa and Asia (especially India), while the *Orobanche* species parasitize several dicotyledonous crops such as tomato, tobacco and sunflower, predominantly in the Mediterranean area, Eastern Europe and in the Middle East. The germination of the seeds of root parasites depends on the receipt of a chemical signal (germination stimulant) present in the rhizosphere of host and some non-host plants. These parasitic flowering plants are entirely dependent on a specific association with the host that provides them with nutrients and water.

The life cycle of the parasitic weeds *Striga* and *Orobanche* is extremely well adapted to their host plants. Each *Striga* and *Orobanche* plant produces thousands of extremely small seeds. The ability of these seeds to remain viable and dormant for many years results in a dramatic increase of the parasite seedbank. The level of infestation may become so great, that normal cereal production is impossible and farmers abandon these fields in search of less infested areas. To date, four germination stimulants (strigolactones) have been isolated *viz*. (+)-strigol (**1**), (+)-sorgolactone (**2**), alectrol (**3**) and (+)-orobanchol (**4**) (Figure 1).

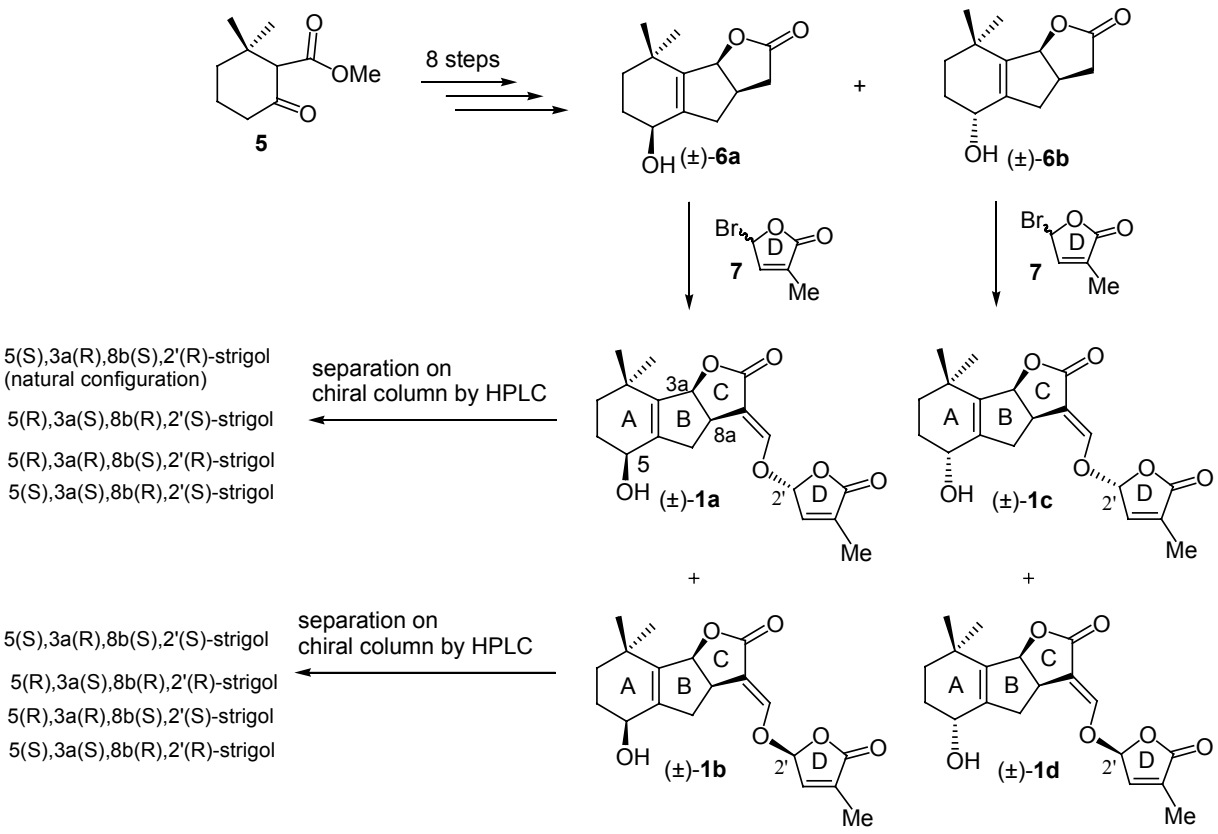
Figure 1



It has been shown that the CD-part of strigolactones is essential for the germination stimulatory activity. Further more, the absolute configuration of these rings also has a significant effect on the biological activity. This led to the hypothesis that the induction of *Striga* and *Orobanche* seed germination proceeds via a receptor mediated mechanism. Association of strigolactone to this receptor will lead to a cascade of reactions resulting in the germination of the seeds. Isolation and purification of the Strigolactone receptor will provide the understanding of the initial stages of *Striga* and *Orobanche* seed germination and enable better control of these parasitic weed pests.

In **chapter 1** the background for the research is given. The problem of parasitic weed pests is described and an overview of the syntheses of germination stimulants is presented. Special attention is given to the synthetic methods utilized in the synthesis of strigol, the first isolated germination stimulant. In addition, a summary of the synthetic methods to obtain enantiopure strigolactones is presented.

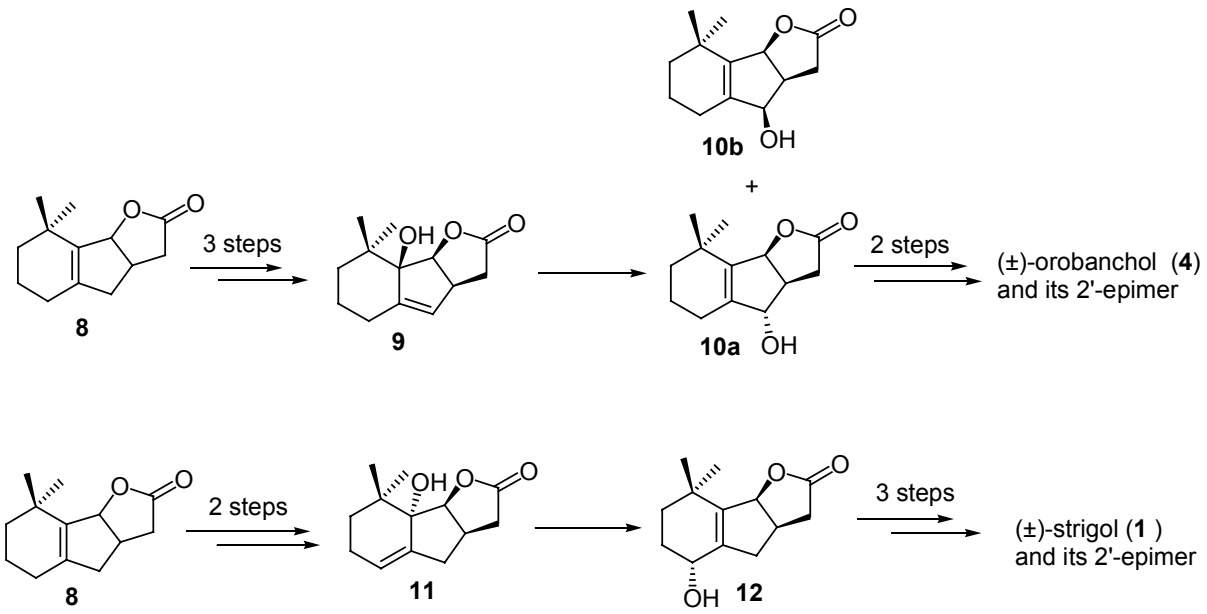
Scheme 1



In **Chapter 2** the synthesis and biological studies of all eight stereoisomers of the naturally occurring germination stimulant strigol (**1**), is described. In the past three decades, strigol has received much attention from synthetic chemists, however, only four out of the eight isomers have been reported. In this chapter, a novel synthesis of all four racemic strigol stereoisomers, all eight isomers, **1(a-d)** and *ent*-**1(a-d)**, is described (Scheme 1). The synthetic strategy involves the preparation of the tricyclic lactone ABC-rings, *rac*-**6a** and *rac*-**6b,** from the readily available keto ester **5** in 8 steps. The lactones were subsequently coupled with the butenolide D-ring (**7)** to obtain all four racemic stereoisomers *rac*-**1(a-d)**. These were subsequently separated on a chiral column to give all eight isomers in enantiopure form.
The bioactivities of all eight stereoisomers of strigol were determined for the seeds of *S. hermonthica*. Strigol with the natural absolute configuration (**1a**) was the most active, whereas, its enantiomer was five time less active at a concentration of 10^{-8} mol/L.

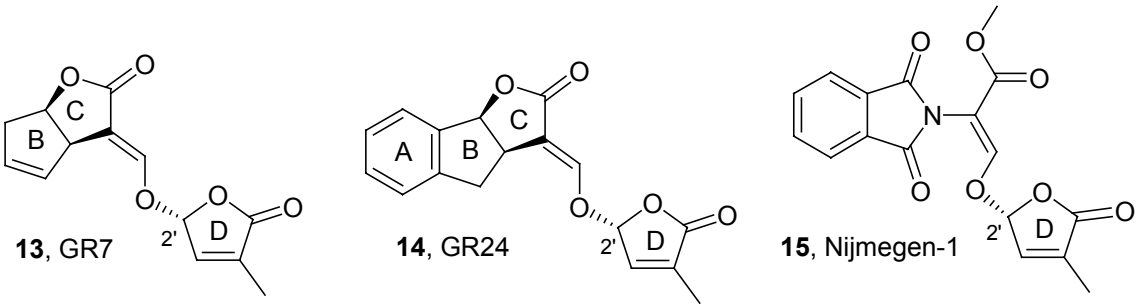
In **Chapter 3** an efficient synthesis of the germination stimulants (±)-orobanchol (**4**) and (±)-strigol (**1**) via an allylic rearrangement of lactones **9** and **11**, is described. This rearrangement was accomplished by treatment of **9** or **11** with trifluoroacetic acid and subsequent alkaline hydrolysis to give the ABC-part of orobanchol **10a** and strigol **12**, respectively. Each of the lactones was coupled to the butenolide D-ring **7** to give (±)-orobanchol and (±)-strigol, respectively (Scheme 2)

Scheme 2

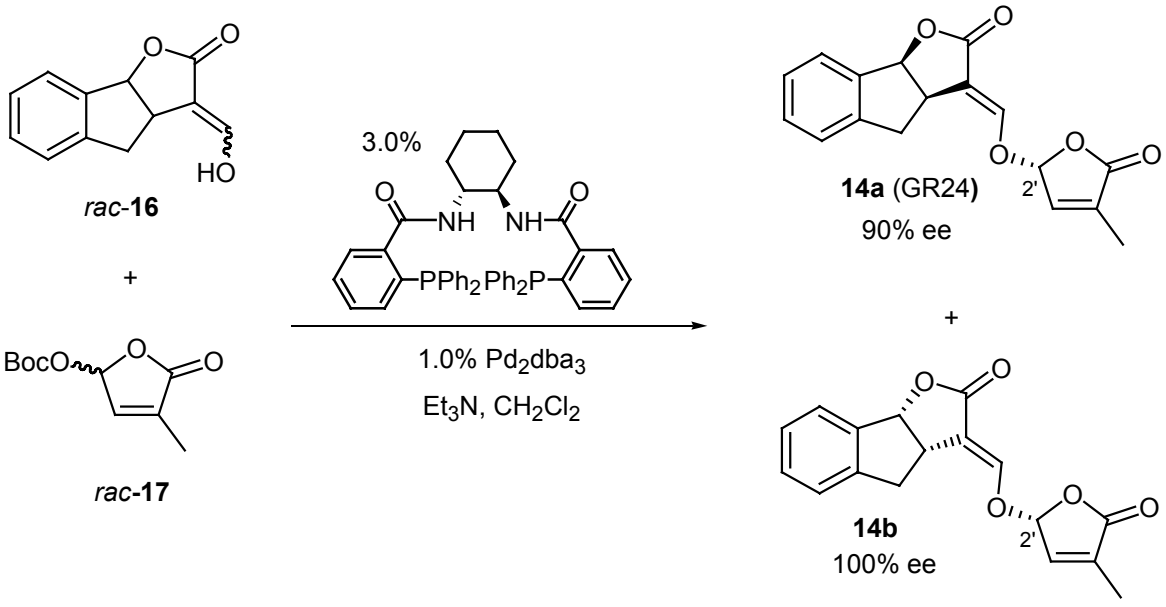


In **Chapter 4** an efficient enantioselective method for the preparation of the strigolactones GR7 (**13**), GR24 (**14**) and Nijmegen-1 (**15**) (Scheme 3), based on a palladium-catalyzed asymmetric coupling, is described. This method is an attractive alternative for the installation of the D-ring with the desired strereochemistry at the C2' position (Scheme 4).

Scheme 3



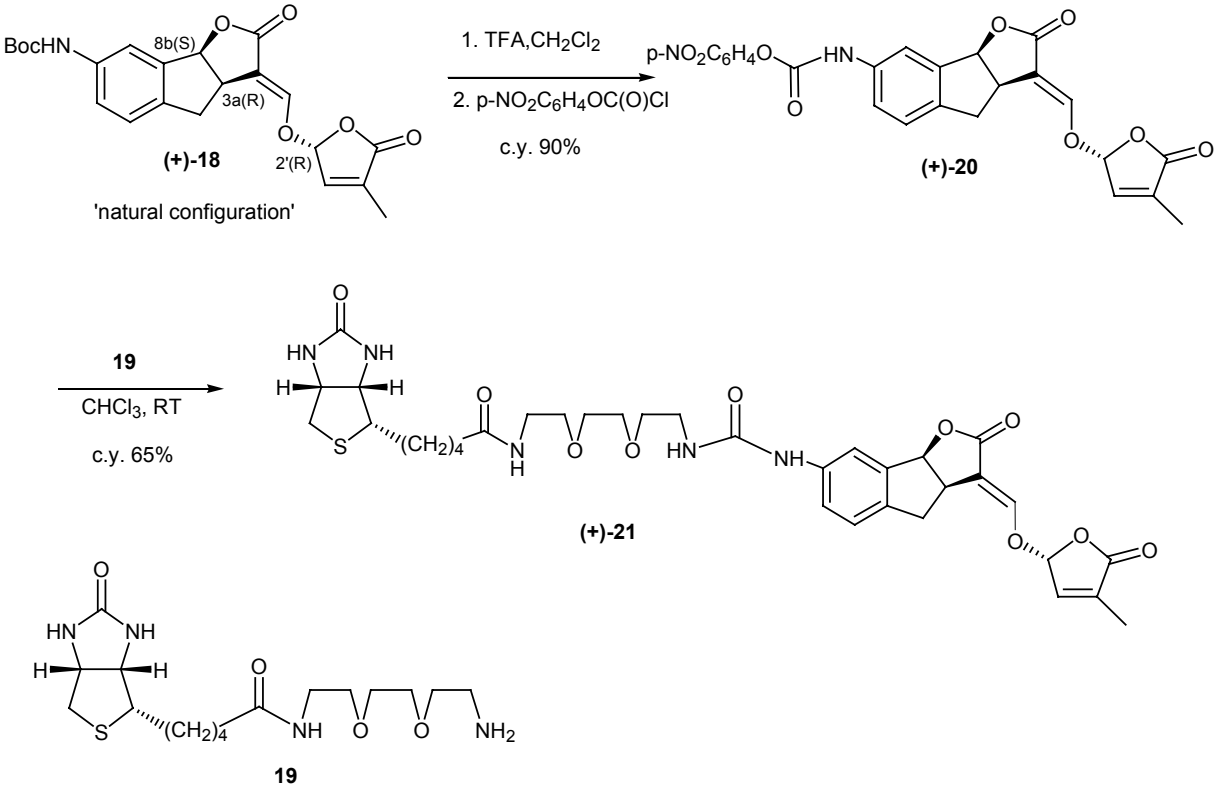
Scheme 4



Chapter 5 is devoted to the synthesis and bioactivity of labelled germination stimulants for the isolation and identification of the strigolactone receptor. A biotin labelled strigolactone analogue was prepared, which was then used for the isolation and purification of the strigolactone receptor by affinity chromatography, using immobilized avidin or streptavidin.

The biotin moiety was attached directly, as well as indirectly via a hydrophilic linker (**19**), to the amino function of enantiopure amino-GR24 ((+)-**18**) (Scheme 5). A second set of labelled stimulants was prepared using amino-GR24, which may enable the identification of the receptor by means of fluorescence correlation spectroscopy, scanning force microscopy or photoaffinity techniques. Bioassays of the labelled stimulants revealed that the germination activity on seeds of *Striga hermonthica* is retained.

Scheme 5



Chapter 6 deals with an attempt to isolate and identify the strigolactone receptor using biotin-labeled stimulant (+)-**21**. The presence of a strigolactone specific binding protein (SLBP) in the insoluble membrane fractions of *Striga* seeds was shown by a dot-blot analysis (Figure 2). Preliminary results with SDS-PAGE showed an enrichment of a 60 kDa protein, isolated from these fractions by affinity purification. (Figure 3, lanes a-d)

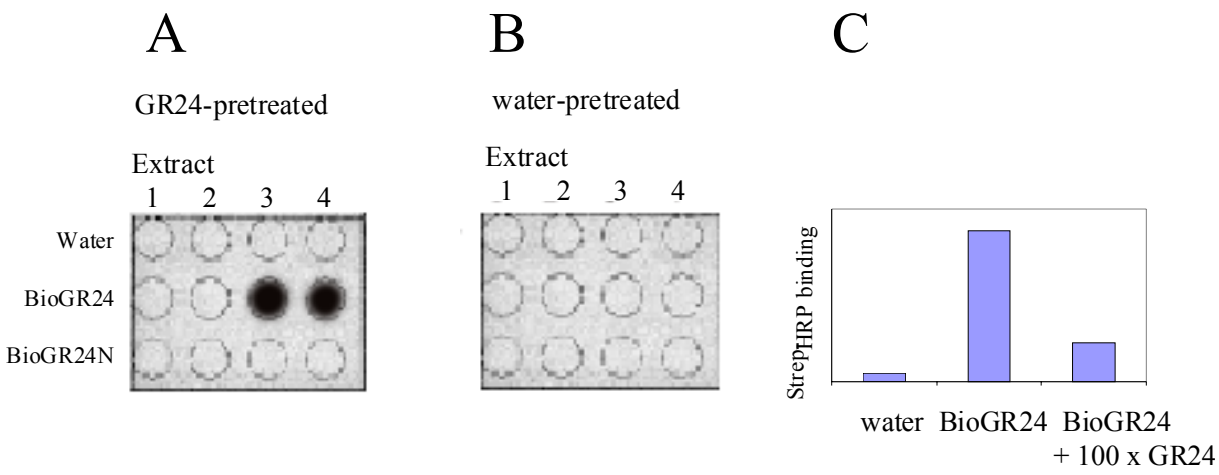


Figure 2 **(A)** Chemoluminescent detection of bound BioGR24 in a dot-blot analysis. Extract 3 and 4 from BioGR24 pretreated seeds showed a signal after incubation with additional BioGR24. **(B)** Chemoluminescent detection of bound BioGR24 in a dot-blot analysis of extracts from water treated seeds (group B) shows no signal. **(C)** Competition of BioGR24 by unlabelled GR24: Chemoluminiscence signal from biotin-bound HRP streptavidin is significantly lowered by the addition of 100 fold molar excess of unlabelled GR24.

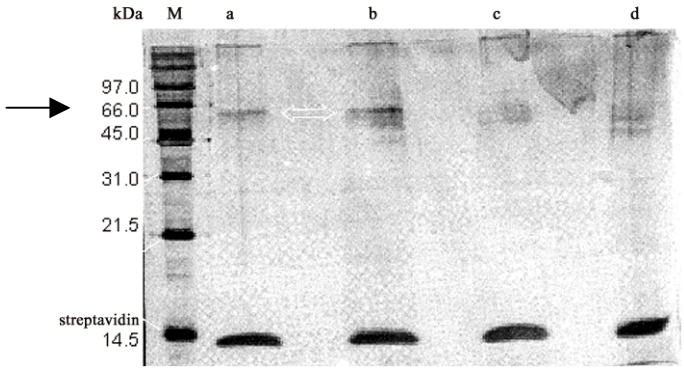


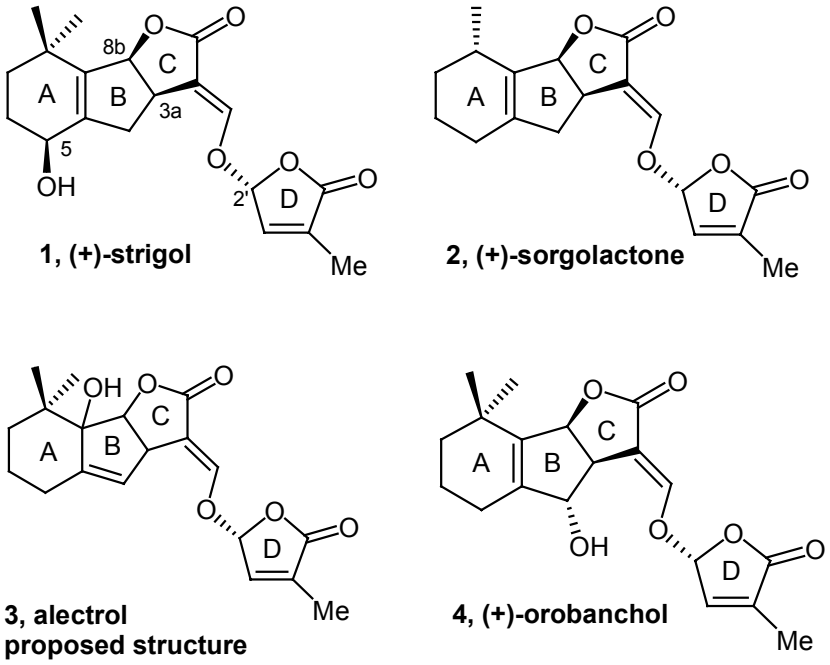
*Figure 3***.** SDS-PAGE of extract 4 (lane a) and extract 3 (lane b) from group A seeds, after affinity purification with streptavidin beads. Similarly, extract 4' (lane c) and 3' (lane d) from group B seeds after affinity purification with streptavidin beads. All lanes were stained with silver. Molecular mass marker: 60 kDa for the SLBP.Group A seeds: pretreated with BioGR24 before grinding; group B seeds: pretreated with water only.

Samenvatting

De onkruiden *Striga* (Scorphulariaceae) and *Orobanche* (Orobanchaceae) zijn planten die parasiteren op de wortels van specifieke gastheerplanten, vaak met dramatische gevolgen voor deze gastheer. Beide onkruiden parasiteren op belangrijke voedselgewassen. *Striga* spp. tast vooral eenzaadlobbigen aan zoals mais, sorghum, gierst en rijst in tropische en subtropische gebieden in Afrika en Azië (vooral India). *Orobanche* spp. parasiteert op een aantal tweezaadlobbigen zoals tomaten, tabak en zonnebloem, hoofdzakelijk in het Middellandse Zeegebied, Oost-Europa en het Midden-Oosten.

Deze parasitaire planten zijn volledig afhankelijk van hun gastheer voor water en voedings stoffen en hun levenscyclus is dus geheel aangepast aan die van hun gastheer. Iedere *Striga* en *Orobanche* plant produceert duizenden zeer kleine zaadjes die jaren lang levensvatbaar blijven en pas zullen ontkiemen zodra een gastheergewas aanwezig is. Het zaad van deze onkruiden ontkiemt namelijk alleen na contact met specifieke chemische verbinding (kiemstimulant) die worden afgescheiden door de wortels van de gastheergewassen. De aantasting van de voedselgewassen kan dermate ernstig zijn dat de oogst volledig verloren gaat. Boeren worden zo gedwongen om andere akkers te zoeken met een geringere besmetting aan onkruidzaden. Tot nu toe zijn vier kiemstimulanten (zgn. strigolactonen) geisoleerd; nl (+)-strigol (**1**), (+)-sorgolacton (**2**), alectrol (**3**) en (+)-orobanchol (**4**) (figuur 1).

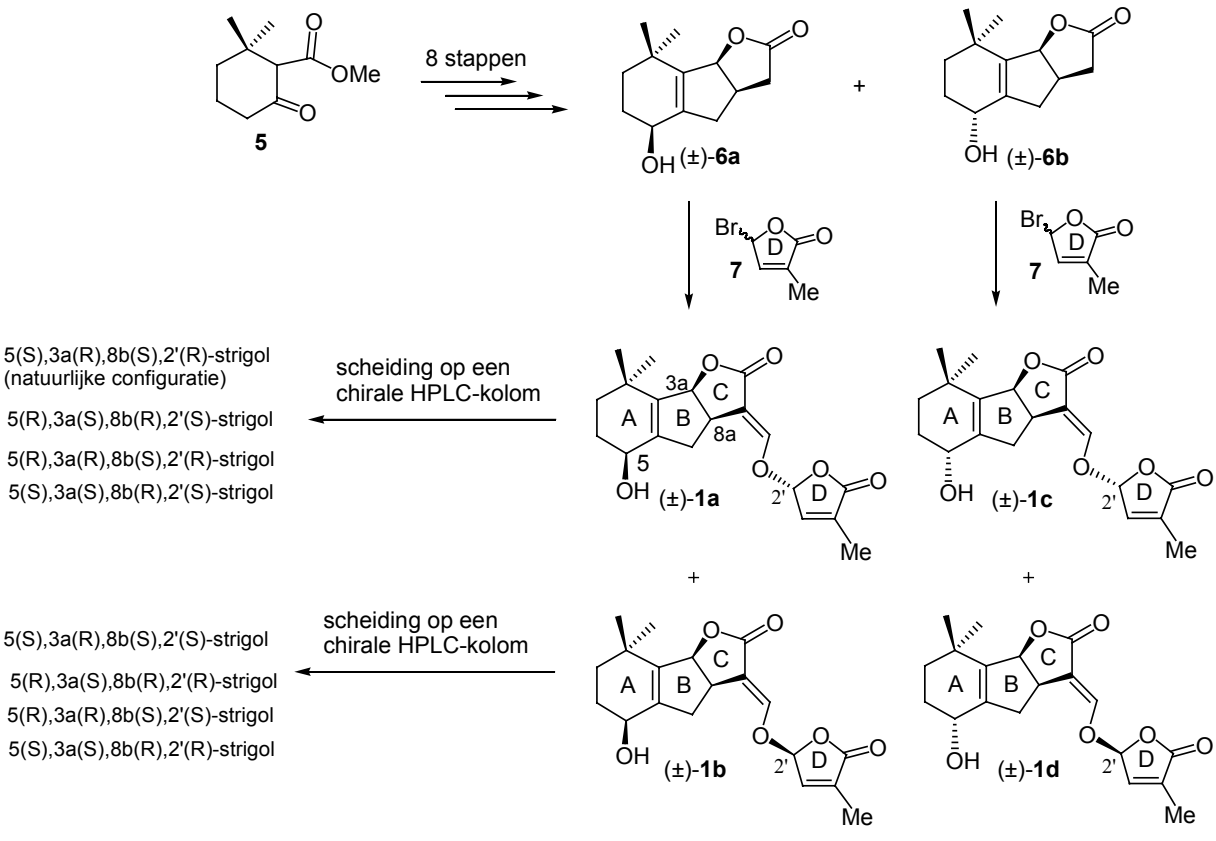
Figuur 1



Eerder onderzoek heeft aangetoond dat het CD-gedeelte van de strigolactonen essentieel is voor hun activiteit als kiemstimulant. Verder is ook gebleken dat de absolute configuratie van dit structuurelement belangrijk is voor de biologische activiteit. Deze waarnemingen hebben geleid tot de hypothese dat de activering van de *Striga* en *Orobanche* zaden verloopt via een receptor-gemedieerd mechanisme. De binding van een strigolacton met deze receptor zou leiden tot een cascade van reacties resulterend in de ontkieming van de zaden van de parasiet. Isolatie en zuivering van deze strigolacton-receptor zou nieuwe inzichten kunnen verschaffen in de eerste stadia van de ontkieming van *Striga* en *Orobanche* zaden en zou kunnen leiden tot betere bestrijdingsmethoden van deze parasitare onkruiden.

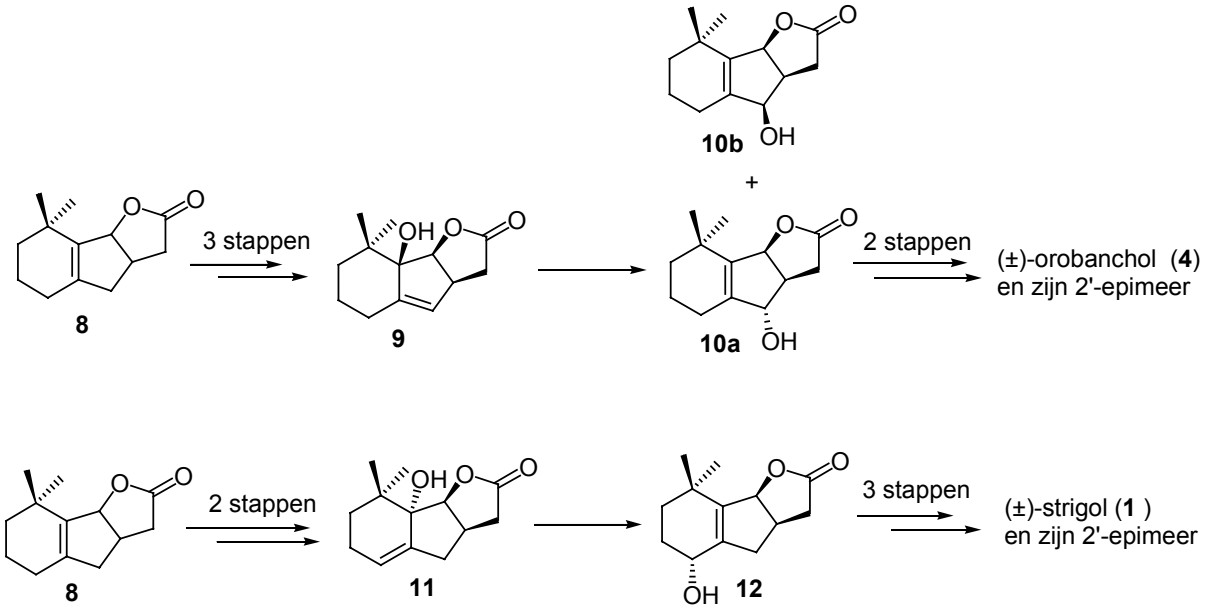
In **Hoofdstuk 1** worden de achtergronden van het onderzoek besproken. Het problem van de parasitaire onkruiden wordt beschreven en er wordt een overzicht van de synthese van kiemstimulanten gegeven. De nadruk ligt hierbij op de synthese van strigol, de eerste kiemstimulant die werd geïsoleerd. Verder wordt een kort overzicht gegeven van methoden voor het verkrijgen van enantiomeerzuivere strigolactonen.

Schema 1



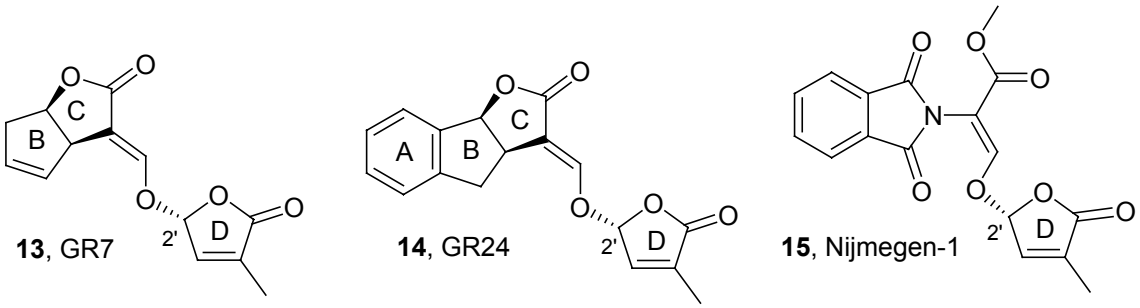
In **Hoofdstuk 2** wordt de synthese en de bepaling van de biologische activiteit van alle acht stereoisomeren van de kiemstimulant strigol (**1**) beschreven. In de afgelopen drie decennia zijn meerdere syntheses van strigol gepubliceerd, echter slechts vier van de acht mogelijke stereoisomeren zijn tot dusver gesynthetiseerd. In dit hoofdstuk wordt een nieuwe synthese van de vier racemische stereoisomeren, en alle acht isomeren **1**(**a-d**) en *ent*-**1**(**a-d**) van strigol beschreven (Schema 1). De strategie omvat de synthese van het tricyclische lactonen *rac*-**6a** en *rac*-**6b**, (ABC-gedeelte), uit de gemakkelijk te bereiden keto-ester **5** in acht stappen. Deze lactonen warden vervolgens gekoppeld aan de butenolide D-ring (**7**) hetgeen leidde tot de vier racemische stereoisomeren *rac*-**1**(**a-d**). Deze isomeren werden vervolgens gescheiden door middel van chirale chromatografie zodat alle acht isomeren in enantiomeerzuivere vorm werden verkregen. De biologische activiteit van deze acht stereoisomeren van strigol werd bepaald voor het zaad van *Striga hermontica*. Strigol met de natuurlijke configuratie (**1a**) vertoonde de hoogste activiteit, terwijl *ent*-(**1a**) een factor vijf minder actief was bij een concentratie van 10^{-8} mol/L.

In **Hoofdstuk 3** wordt een efficiente synthese van de kiemstimulanten (±)-orobanchol (**4**) en (±)-strigol (**1**) via een allylische omlegging van de lactonen **9** en **11** beschreven. Deze omlegging werd bewerkstelligd door behandeling van **9** of **11** met trifluorazijnzuur gevolgd door basische hydrolyse hetgeen resulteerde in de vorming van respectievelijk het ABC-gedeelte van orobanchol **10a** en strigol **12**. Beide lactonen werden vervolgens gekoppeld aan de butenolide D-ring hetgeen respectievelijk (±)-orobanchol (**4**) en (±)-strigol (**1**) opleverde (Schema 2).Schema 2

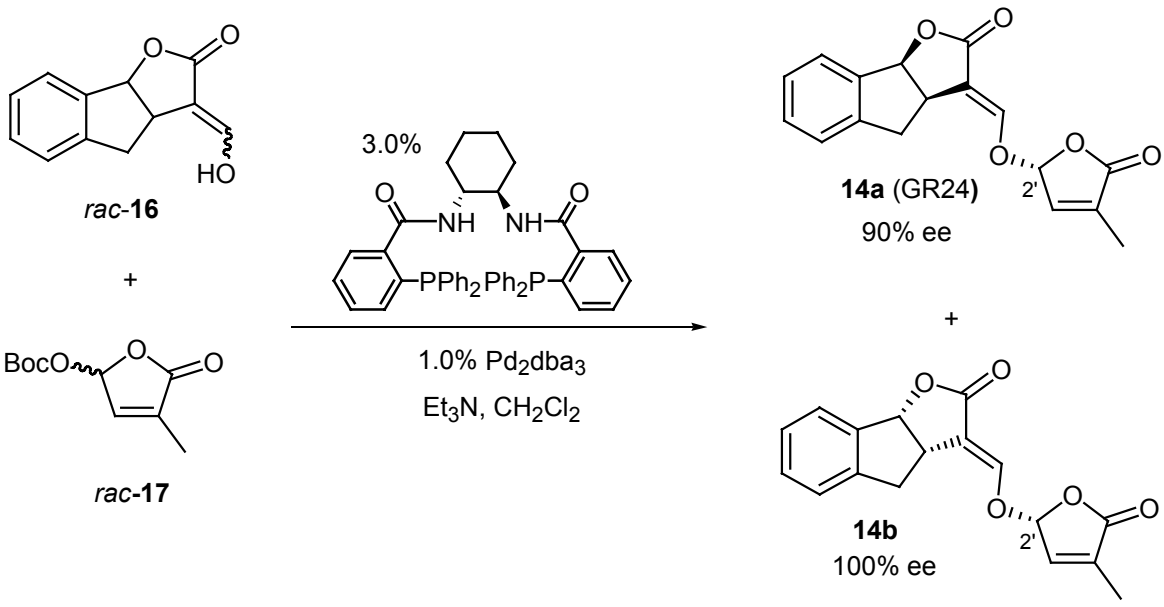


Hoofdstuk 4 handelt over een efficiente enantioselectieve bereiding van de strigolactonen GR7 (13), GR24 (14) en Nijmegen-1 (15) (Schema 3), gebaseerd op een palladium-gekatalyseerde asymmetrische koppeling. Deze methode is een aantrekkelijk alternatief voor de koppeling van de D-ring met de gewenste stereochemie op de C2'-positie te verkrijgen (Schema 4).

Schema 3



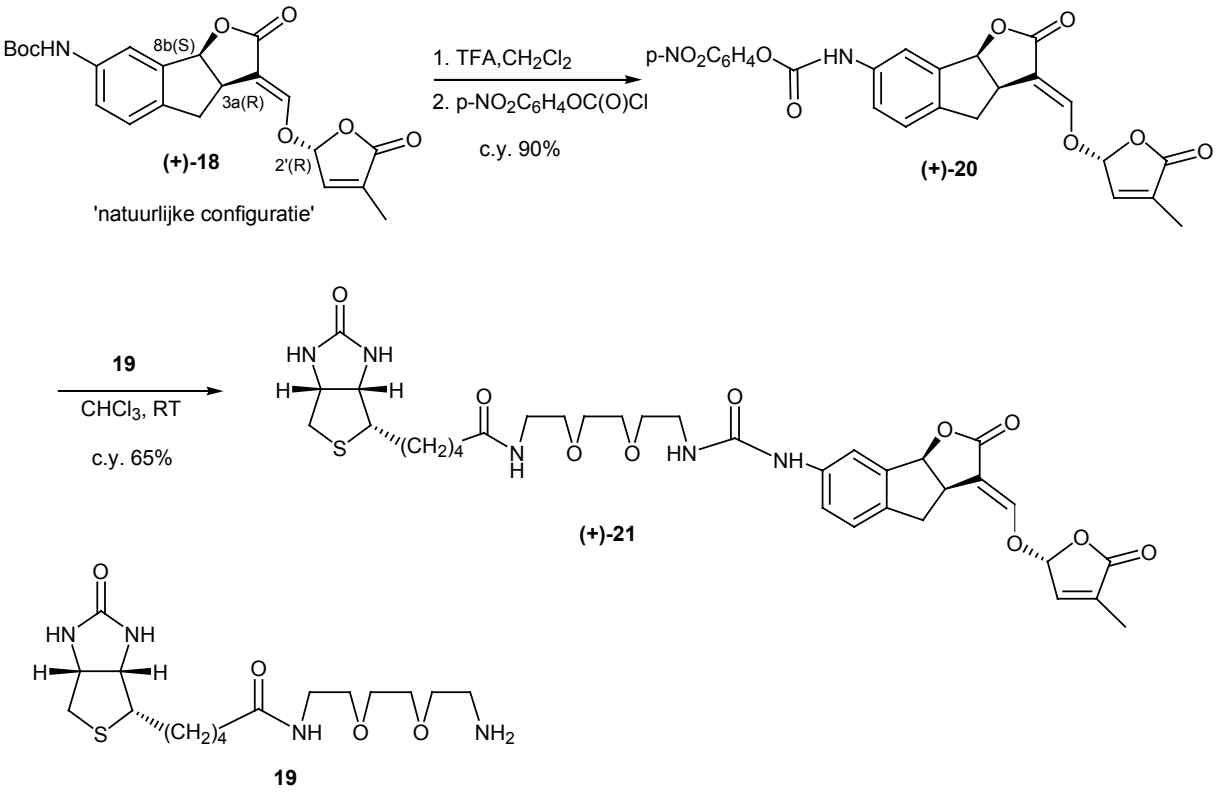
Schema 4



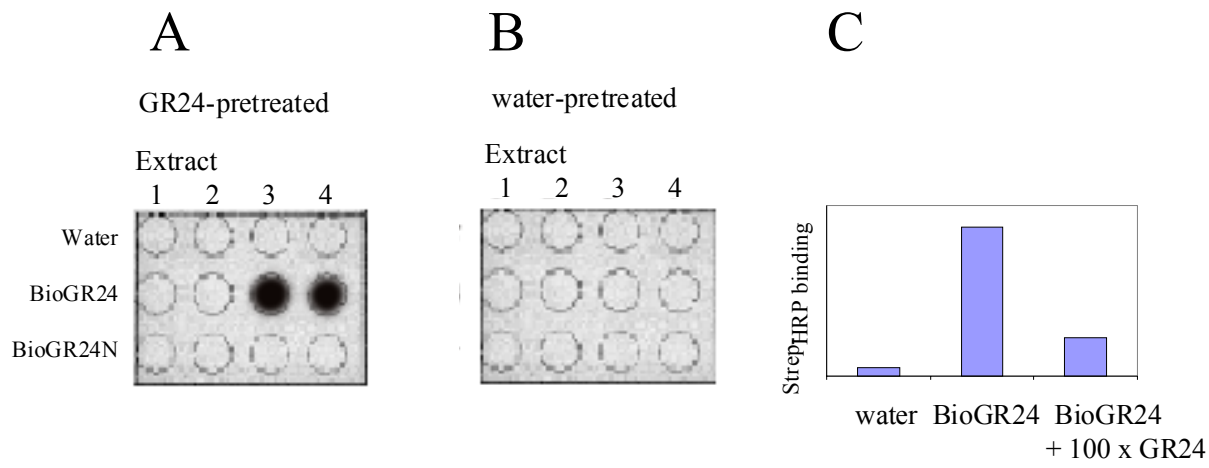
Hoofdstuk 5 is gewijd aan de synthese en biologische activiteit van gelabelde kiemstimulanten voor de identificatie en isolatie van de strigolactonreceptor. Een strigolacton gelabeld met biotine werd gesynthetiseerd en vervolgens gebruikt voor de isolatie en zuivering van de strigolactonreceptor door middel van affiniteitschromatografie,

gebruikmakend van geïmobiliseerd avidine of streptavidine. De biotine-eenheid was zowel direct als indirect via een hydrofobe linker, (**19**), verbonden met enantiomeerzuiver amino-GR24 ((+)-**18**)) (Schema 5). Een tweede set gelabelde kiemstimulanten werd bereid uitgaande van amino-GR24. Deze derivaten zouden de identificatie van de receptor mogelijk kunnen maken door middel van fluorescentie-correlatie-spectroscopie, scanning-force-microscopie of foto-affiniteitstechnieken. Bioassays van de gelabelde stimulanten toonden aan dat kiemingsactiviteit voor zaden van *Striga hermontica* behouden bleef.

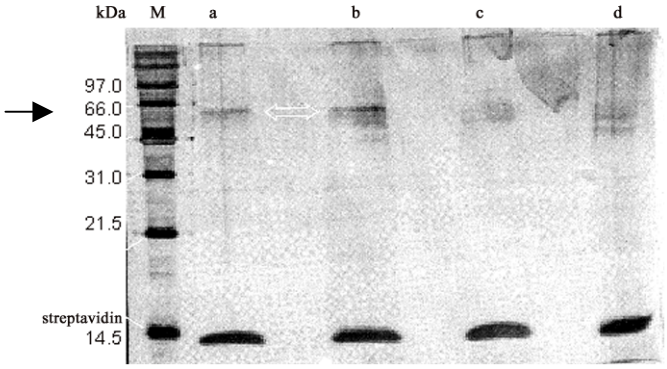
Schema 5



In **hoofdstuk 6** wordt een poging tot isolatie en identificatie van de strigolacton receptor gebruikmakend van de biotine-gelabelde stimulant (+)-**21** beschreven. De aanwezigheid van een 'strigolactone specific binding protein' (SLBP) in de onoplosbare membraanfracties van *Striga* zaden werd aangetoond door middel van een 'dot-blot' analyse (Figuur 2). De voorlopige resultaten met SDS-PAGE-techniek lieten een verrijking zien van een 60 kDa eiwit dat was geisoleerd uit de bovengenoemde fracties met behulp van de affiniteits-zuiveringsmethodiek (Figuur 3, rijen a-d).



Figuur 2 (**A**) Chemiluminescente detectie van gebonden BioGR24 in een dot-blot analyse. Extract 3 en 4 van met BioGR24 voorbehandelde zaden toonden een signaal na incubatie met meer BioGR24. (**B**) Chemiluminescente detectie van gebonden BioGR24 in een dot-blot analyse van extracten van met water voorbehandelde zaden (groep B) geeft geen signaal. (**C**) Competitie van BioGR24 met ongelabeld GR24: Het chemiluminescente signaal van biotine gebonden HRP streptavidine wordt significant verlaagd door toevoeging van honderdvoudige overmaat ongelabeld GR24.



Figuur 3 SDS-PAGE van extract 4 (kolom a) en extract 3 (kolom b) van groep A zaden, na affiniteitszuivering met streptavidineparels. Evenzo, extract 4' (kolom c) en extract 3' (kolom d) van groep B zaden na affiniteitszuivering met streptavidineparels. Alle kolommen werden gekleurd met zilver. Molmassa marker: 60 kDa voor de SLBP. Groep A zaden werden voorbehandeld met BioGR24 voor het vermalen; groep B zaden werden alleen met zuiver water voorbehandeld.

List of Publications

S. Braverman, M. Cherkinsky, L. Kerdova and A. Reizelman. A Novel Synthesis of Isocyanate and Ureas via β-elimination of Haloform. *Tetrahedron Lett*. **1999**, *40*, 3235-3238.

A. Reizelman, M. Scheren, G.H.L. Nefkens and B. Zwanenburg. Synthesis of All Eight Stereoisomers of the Germination Stimulant Strigol. *Synthesis* **2000**, 1944-1951.

A. Reizelman and B. Zwanenburg. Synthesis of the Germination Stimulants (±)-Orobanchol and (±)-Strigol via an Allylic Rearrangement. *Synthesis* **2000**, 1952-1955.

B. Zwanenburg and A. Reizelman. En Route to the Isolation and Characterization of the Strigolactone Receptor Using Biotin Labbeled Strigolactone Analogues. Proceedings of the 7th International Parasitic Weed Symposium, A. Fer, P. Thalouarn, D.M. Joel, L.J. Musselman, C. Parker and J.A.C. Verkleij, Eds., Nantes, France, **2001**, 102-105.

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A. Reizelman, S.C.M. Wigchert, C. del-Bianco and B. Zwanenburg. Synthesis and Bioactivity of Labelled Germination Stimulants for the Isolation and Identification of the Strigolactone Receptor. *Org. Biomol. Chem*. **2003**, *6*, 950.

Oral Presentations and Posters

- *A new approach to the synthesis of the germination stimulant strigol.* Anat Reizelman and B. Zwanenburg. Poster communication in the international symposium on "The impact of Organic Synthesis on Drug Discovery", Sep. 6-8, 1998, University of Heidelberg, Germany.

- *A new approach to the synthesis of the germination stimulant strigol.* Anat Reizelman and B. Zwanenburg. University of Sydney, 1999, Australia, Oral Communication.

- *A new approach to the synthesis of the germination stimulant strigol.* Anat Reizelman and B. Zwanenburg.- Bologna/ Nijmegen Minisymposium VI (BONYMI-VI) , 1998, Nijmegen, The Netherlands, Oral Communication.

- Synthetic Studies Toward Alectrol, a Germination Stimulant. A. Reizelman, G. Anilkumar, and B. Zwanenburg. Poster communication in the national symposium on "Design and Synthesis in Organic Chemistry", Oct. 4-5, 1999, (P. 3) Lunteren, The Netherlands.

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Curriculum Vitae

Anat Reizelman-Lucassen was born on 10th of June 1965 in Beer-Sheva, Israel. Between the years 1986-1988 she studied at, and graduated from the technical school in the department of analytical chemistry in Beer-Sheva. Soon thereafter she started her B.Sc in chemistry at the University of Ben-Gurion, located in the same city. After four years of study, she joined the department of organic chemistry of the Bar-Ilan University to study for her M.Sc under the supervision of prof. dr. S. Braverman, submitting her M.Sc thesis in 1996. From December 1996 until March 1997 she was at the department of Organic Chemistry of the University of Nijmegen for a three-month research period on the *Striga*-project under the supervision of prof. dr. B. Zwanenburg. From April 1997 until August 2001 she was a PhD student in the same department. The results of this research period are described in this thesis. From December 2001 till November 2002 she worked as postdoctoral research fellow at the Weizmann Institute of Science in Rehovot (Israel), in the group of Dr. Dan Tawfik on directed evolution of phosphotriesterases. From November 2002 she is working as a Research Chemist at "Compugen Ltd." in Ashkelon (Israel).